The Interplay Between Hypoxia (HIF) and Inflammation (NF-κB) in Colon Cancer

Dissertation

zur

Erlangung der naturwissenschaftlichen Doktorwürde

(Dr. sc. nat.)

vorgelegt der

Mathematisch-naturwissenschaftlichen Fakultät

der

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Zürich, 2014

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1. Abbreviation list

AhR	aryl hydrocarbon receptor
Ang	angiopoietin
AOM	azoxymethane
AP	activator protein
APC	adenomatous polyposis coli or anaphase promoting complex or antigen presenting cell
ARNT	aryl hydrocarbon receptor nuclear translocator
ATM	ataxia telangiectasia mutated
ATP	adenosine triphosphate
BAFF	B cell activating factor of the TNF family
BCL	B-cell lymphoma
bHLH	basic helix-loop-helix
BNIP	BCL-2/adenovirus E1B 19 kDa interacting protein
CA	carbonic anhydrase
CAC	colitis-associated cancer
cAMP	cyclic adenosine monophosphate
CBP	CREB-binding protein
CCL	chemokine (C-C motif) ligand
CD	cluster of differentiation or Crohn`s disease
CDK	cyclin-dependent kinase
cFLIP	cellular FLICE-like inhibitory protein
cIAP	cellular inhibitor of apoptosis
CIN	chromosomal instability
CK	casein kinase
COX	cyclooxygenase
CPG	central pattern generator
CRC	colorectal cancer
CREB	cAMP responsive element binding protein

CXCL	chemokine (C-X-C motif) ligand
CXCR	chemokine (C-X-C motif) receptor
DC	dendritic cells
DMOG	dimethyloxalylglycine
DSB	double-strand break
dsRNA	double-stranded RNA
DSS	dextran sodium sulfate
DUB	deubiquitinase
ECM	extracellular matrix
EGF	epidermal growth factor
EGLN	egl nine homolog
EMT	epithelial-to-mesenchymal transition
EPAS	endothelial PAS domain-containing protein
FAP	familial adenomatous polyposis
FIH	factor inhibiting HIF
GLUT	glucose transporter
GM-CSF	granulocyte/macrophage colony-stimulating factor
Gro	growth-regulated protein
HBS	HIF binding site
HIF	hypoxia inducible factor
HIV	human immunodeficiency virus
HNPCC	hereditary non-polyposis colon cancer
HPV	human papillomavirus
HRE	hypoxia response element
IBD	inflammatory bowel disease
ICAM	intercellular adhesion molecule
IFN	interferon
IGF	insulin-like growth factor
IKK	inhibitor of kappa B kinase
IL	interleukin

iNOS	inducible nitric oxide synthase
IRAK	interleukin-1 receptor associated kinase
IRF	interferon regulatory factor
ITF	intestinal trefoil factor
IκB	inhibitor of kappa B
LBP	LPS binding protein
LDHA	lactate dehydrogenase A
LPS	lipopolysaccharide
LRR	leucine-rich repeat
MAL	MyD88-adapter-like
MAPK	mitogen-activated protein kinase
MCP	monocyte chemotactic protein
M-CSF	macrophage colony-stimulating factor
MDR	multidrug resistance
MDSC	myeloid-derived suppressor cells
MMP	matrix metalloproteinase
MSH	MutS protein homolog
MSI or MIN	microsatellite instability
MyD88	myeloid differentiation primary response protein 88
NEMO	NF-κB essential modulator
NES	nuclear export signal
NF-κB	nuclear factor kappa B
NIK	NF-κB-inducing kinase
NK	natural killer
NLS	nuclear localization signal
NO	nitric oxide
ODD	oxygen-dependent degradation
OTU	ovarian tumour
OTUB	OTU domain, ubiquitin aldehyde binding
PAS	Per-Arnt-Sim

PDGF	platelet-derived growth factor
PDK	pyruvate dehydrogenase kinase
Per	period circadian protein
PET	positron emission tomography
PGK	phosphoglycerate kinase
PHD	prolyl-4-hydroxylase domain
PI_3K	phosphoinositide 3-kinase
PKC	protein kinase C
PMN	polymorphonuclear
PTGS	prostaglandin-endoperoxide synthase
RHD	Rel homology domain
RIP	receptor-interacting protein kinase
ROS	reactive oxygen species
SDF	stromal cell-derived factor
shRNA	short hairpin RNA
SIGIRR	single immunoglobin IL-1R-related
SIM	single-minded protein
siRNA	small interfering RNA
SKP	S-phase kinase-associated protein
ssRNA	single-stranded RNA
STAT	signal transducer and activator of transcription
TAB	TGF-beta activated kinase 1 (MAP3K7) binding protein
TAD	transactivation domain
TAK1	TGF-beta activated kinase 1
TAM	tumour-associated macrophage
TAN	tumour-associated neutrophil
TANK	TRAF family member-associated NF-κB activator
TBK	TANK-binding kinase
TGF	transforming growth factor
Tie	tunica interna endothelial cell kinase

TIR	Toll/interleukin-1 receptor
TIRAP	TIR domain-containing adapter protein
TLR	Toll-like receptor
TNBS	2,4,6-trinitrobenzene sulfonic acid
TNF	tumour necrosis factor
TNFAIP	tumour necrosis factor alpha-induced protein
TNFR	tumour necrosis factor receptor
TRADD	TNFR1-associated death domain protein
TRAF	tumour necrosis factor receptor-associated factor
TRAM	TRIF-related adaptor molecule
TRIF	Toll/interleukin-1 receptor domain-containing adapter protein inducing interferon beta
UBC	ubiquitin conjugating enzyme
UC	ulcerative colitis
uPA	urokinase-type plasminogen activator
uPAR	urokinase-type plasminogen activator receptor
VCAM	vascular cell adhesion molecule
VEGF	vascular endothelial growth factor
VHL	von Hippel-Lindau

2. Zusammenfassung

Das kolorektale Karzinom ist die zweithäufigste Krebserkrankungen bei Frauen und die dritthäufigste bei Männern. Hypoxie und Entzündung sind untrennbare Merkmale von Krebserkrankungen und beeinflussen dieselben kritischen Prozesse der Krebsentwicklung wie z.B. das Überleben der Zellen, Zellinvasion, Angiogenese, Metastasierung und Therapieresistenz. Hypoxie und Entzündung sind beides Merkmale von Krebserkrankungen, einschliesslich dieses Krebstyps. Die Hauptakteure der zellulären Anpassung an Hypoxie sind die Hypoxie-induzierbaren Faktoren 1 und 2 (HIF-1 und HIF-2). Beide setzen sich aus einer sauerstoff-empfindlichen Untereinheit (HIF-1α oder -2α) und dem konstitutiv exprimierten Bindungspartner HIFβ zusammen. HIF-1 wird universell exprimiert. HIF-2 zeigt hingegen eine Zelltyp-spezifische Expression. Unter normalen Sauerstoff-bedingungen werden die HIF-α Untereinheiten nach der Hydroxylierung durch Prolyl-4-Hydroxylase (PHD)-Proteine rasch durch das Proteasom abgebaut. Hypoxie führt durch die Inhibierung der PHD-Enzyme zur Stabilisierung von HIF-α. Infolgedessen bilden HIF-α und HIFβ ein funktionelles Heterodimer, welches die Zielgene aktiviert. Der primär für die Entzündungsreaktion verantwortliche Transkriptionsfaktor NF-κB besteht aus zwei Untereinheiten, vor allem p65/RelA und p50. Unter unstimulierten Bedingungen wird NF-κB von inhibierenden Proteinen (hauptsächlich IκBα) im Zytoplasma zurückgehalten. Eine proinflammatorische Stimulation führt zur Phosphorylierung und Abbau von IκBα. Durch Translokation gelangt NF-κB dann in den Zellkern und aktiviert dort die Zielgene. Aktivierung von HIF-1 wie auch NF-κB werden dabei mit einer schlechteren Prognose für die Patienten in Verbindung gebracht. Darüber hinaus besteht eine gut bekannte Assoziation zwischen einer chronisch-entzündlichen Darmerkrankung und einem erhöhten Malignitätsrisiko. Im Allgemeinen wird davon ausgegangen, dass es auf mehreren Ebenen zu Interaktionen zwischen dem Hypoxie- und dem Entzündungssignalweg kommt, wie etwa bei der direkten transkriptionellen Aktivierung von HIF-1α durch NF-κB oder der Hypoxie-vermittelten Aktivierung von NF-κB. Das Ziel dieser Arbeit bestand daher darin, eine umfassende Analyse der Relevanz dieser beiden Signalwege beim kolorektalen Karzinom, wie auch eine Untersuchung eines möglichen Wechselspiels zwischen diesen beiden Signalwegen in diesem Krebsmodell vorzunehmen. Die

Hypoxieexposition von MC-38 Zellen führt zur Stabilisierung des HIF-1α Proteins und zur Aktivierung der bekannten HIF-Zielgene *Glut1*, *Ca9* und *Phd3* auf mRNA-Ebene. MC-38 Zellen zeigen eine robuste Antwort des Transkriptionsfaktors NF-κB auf einen entzündungsfördernden Stimulus. Die Behandlung dieser Zellen mit LPS führt zur Translokation von NF-κB in den Zellkern und zur Aktivierung der Zielgene *Tnfa*, *Il6* und *Cox2*. Dieser Effekt der LPS-Stimulierung wird noch verstärkt, wenn die Zellen in 3D (in hängenden Tropfen) kultiviert werden. Der HIF Signalweg (das HIF-1α Transkript und der Proteinspiegel, wie auch die *Glut1*, *Ca9* und *Phd3* Genexpression) wird durch die proinflammatorische Stimulation der MC-38 Zellen mit LPS nicht beeinflusst, während der NF-κB Signalweg aktiviert wird. Auch in mehreren getesteten kolorektalen Krebszelllinien konnte keine transkriptionelle Regulation der HIF-1α mRNA nach einer Behandlung mit LPS festgestellt werden. Ferner reagieren MC-38 Zellen unter Hypoxie in dem sie den HIF Signalweg aktivieren, was aber nicht zu einer stärkeren NF-κB Aktivität führte. Interessanterweise ist die hypoxische Induktion sowohl des HIF-1α Proteins wie auch der GLUT-1, CAIX und PHD3 Zielgene unverändert, wenn die basale NF-κB Aktivität fehlt, was durch einen Knock-down von p65/RelA in MC-38 Zellen gezeigt werden konnte. Auch zeigt die LPS Behandlung von MC-38 Zellen mit einem HIF-1α Knock-down, dass die basale HIF-1α Expression irrelevant für die nukleäre Translokation von NF-κB, wie auch für die Expression von TNF-α und IL-6 ist. Eine RNA Sequenzierungsanalyse der Genregulation unter verschiedenen Sauerstoffbedingungen und/oder LPS Behandlung zeigte eine profunde gegenseitige Abhängigkeit der beiden Signalwege. 58% der unter Hypoxie heraufregulierten Gene benötigen für ihre Induktion HIF-1α, wie auch NF-κB, während nur ungefähr 11% der Gene, die durch eine LPS Behandlung heraufreguliert werden, von beiden Transkriptionsfaktoren abhängig sind. Ein anderes unerwartetes Resultat der kombinierten Hypoxie- und LPS Exposition war die Hypoxie vermittelte Abschwächung der Antwort der MC-38 Zellen auf die LPS Behandlung. Die Gesamtanzahl der Gene, welche durch eine LPS-Behandlung induziert werden, verringerte sich in der Hypoxie um etwa 50%, verglichen mit den ebenfalls LPS behandelten Zellen in Normoxie. Die nachfolgenden Experimente zeigten, dass die Hypoxie die LPS-stimulierte IκBα Abnahme und die Translokation von NF-κB in den Zellkern beeinträchtigt. Dieser Effekt ist von HIF-1α unabhängig. Der höhere IκBα Menge erklärt wenigstens

teilweise die verminderte Entzündungsantwort in MC-38 Zellen unter Hypoxie. Schliesslich zeigte die RNA Sequenzierung eine HIF-1α Abhängigkeit der Inhibition der LPS Induzierbarkeit von verschiedenen gut bekannten NF-κB Zielgenen (*Ccl20*, *Cxcl5*, *Csf2* und *Tnfa*). Diese Resultate wurden durch unabhängige Validierung mittels RT-qPCR bestätigt. Die oben genannten Gene sind in der Rekrutierung und Aktivierung von Leukozyten involviert. Deshalb ist es notwendig eine Analyse dieses Effektes von HIF-1 (sowie auch der neu identifizierten HIF-1 regulierten Entzündungsmediatoren) durchzuführen, um einen Einblick in die *in vivo* Relevanz von HIF-1 als gestaltender Faktor für die Entzündungsreaktion in diesem Krebstyp zu erhalten.

3. Summary

Colorectal cancer is the second most common malignancy in women and the third in men. Hypoxia and inflammation are two inseparable hallmarks of cancer influencing the same crucial processes in carcinogenesis, including cell survival, invasion, angiogenesis, metastasis and resistance to therapy. The main players involved in the cellular response to hypoxia are hypoxia-inducible factor-1 and -2 (HIF-1 and HIF-2) composed of the oxygen-labile subunit HIF-1α or HIF-2α, respectively, and their constitutively expressed common interaction partner HIFβ. Whereas HIF-1 is expressed ubiquitously, HIF-2 displays a cell type-specific expression pattern. In normal oxygen conditions HIF-α subunits are rapidly degraded by proteasomes. Normoxic disassembly of HIF-α is associated with the activity of prolyl-4-hydroxylase domain (PHD) enzymes. Hypoxia leads to the stabilization of HIF-α by inhibiting PHD enzyme activity. HIF-α subunits form functional heterodimers with HIFβ, subsequently activating HIF target genes. The main transcription factor involved in the classically activated inflammatory response is the NF-κB heterodimer composed of p65/RelA and p50 subunits. Under unstimulated conditions, NF-κB is retained in the cytoplasm by interacting with inhibitory proteins (mainly IκBα). Proinflammatory stimulation of the cell results in the phosphorylation and degradation of IκBα. Liberated NF-κB dimers translocate to the nucleus and activate target genes. Activation of both, HIF-1 and NF-κB has been linked to poorer colorectal carcinoma patient prognosis. Furthermore, there is a well established association between inflammatory bowel disease and increased risk of developing colorectal cancer. There are several levels of established interactions between hypoxic and inflammatory pathways, including direct transcriptional activation of HIF-1α by NF-κB, and hypoxia-mediated activation of NF-κB. Therefore, the aim of this work was a comprehensive analysis of the relevance of both pathways in colon cancer cells as well as the study of their possible interplay in this cancer model. Hypoxic exposure of MC-38 cells resulted in the stabilization of HIF-1α protein and activation of its classical target genes *Glut1*, *Ca9* and *Phd3*. MC-38 cells also displayed a robust response of the transcription factor NF-κB to proinflammatory stimulation. Treatment of MC-38 cells with LPS led to nuclear NF-κB translocation and activation of its target

genes *Tnfa*, *Il6* and *Cox2*. The effect of LPS stimulation of MC-38 is further enhanced in cells grown in 3D hanging drop culture conditions. The HIF pathway (HIF-1α transcript and protein levels, as well as *Glut1*, *Ca9* and *Phd3* gene expression) is unaltered under conditions of proinflammatory treatment with LPS in MC-38 cells, despite activation of NF-κB signalling. In line, no transcriptional regulation of HIF-1α mRNA upon treatment with LPS could be observed in a panel of human colon cancer cell lines tested. Furthermore, although MC-38 cells respond to hypoxia by upregulating the HIF pathway, hypoxic treatment does not lead to an increase in NF-κB levels or activity. Interestingly, the hypoxic induction of the HIF-1α protein, as well as GLUT-1, CAIX and PHD3 transcripts is independent of NF-κB, as studied in MC-38 cells with p65/RelA knock-down. Similarly, basal HIF-1α expression was irrelevant for NF-κB nuclear translocation and for the expression of TNF-α and IL-6 following the LPS treatment, as addressed in MC-38 cells with HIF-1α knock-down. RNA deep sequencing was performed to gain a further insight into complexity of combined hypoxic and proinflammatory treatment. Exposure of MC-38 cell to hypoxia and/or LPS revealed profound mutual dependence of both pathways. An unexpectedly high proportion of 58% of hypoxically upregulated genes required both HIF-1α and NF-κB for their induction, whereas LPS-mediated upregulation of only about 11% of genes needed both transcription factors. Another unexpected result of combined hypoxic and inflammatory treatment was the hypoxia-mediated attenuation of molecular response to LPS. The total number of genes induced by the combined treatment with LPS and hypoxia was decreased by about 50% compared to LPS alone. The follow-up experiments showed that hypoxia impairs LPS-stimulated IκBα levels decrease leading to attenuated nuclear translocation of NF-κB, an effect that was independent of HIF-1α. Inhibiting the decrease in IκBα at least in part explains the diminished inflammatory response in MC-38 cells in hypoxia. Finally, RNA deep sequencing, confirmed by independent RT-qPCR validation, revealed an increase in the LPS-inducibility of several well known NF-κB target genes (*Ccl20*, *Cxcl5*, *Csf2* and *Tnfa*) in the absence of HIF-1α. Those genes are known to be involved in the recruitment and activation of leukocytes. Future analysis of the effect of HIF-1 (and each of newly identified HIF-1-regulated inflammatory mediators) aiming at investigating the *in vivo* relevance of HIF-1 as a factor shaping the inflammatory component in this type of malignancy is required.

4. Introduction

4.1. Cancer

14.1 million new cancer cases were diagnosed in 2012 and cancer accounted for 8.2 million deaths worldwide in the same year. Among them, tumours of lung, liver, stomach, colorectum and prostate are major cause of cancer-related deaths in men, and of breast, lung, colorectum, cervix uteri and stomach in women (*1*).

Research on cancer has it beginnings in the late XIX century when Johannes Mueller, a German microscopist, described the cellular nature of cancer. This discovery started the long study on the differences between normal and cancerous tissues (*2*).

4.1.1. What is tumour

It is crucial to distinguish non-pathologic conditions, resulting from increased demand applied on a given organ or its hormonal stimulation, leading to hypertrophy (increased size of the cells, e.g. muscle cells in athletes) or hyperplasia (increased number of cells, e.g. of endometrium in female monthly cycle) from neoplasia (the formation of tumour). Hyperthrophy and hyperplasia are stimulus-dependent and cease when that cause/stimulus is withdrawn (*2*). In turn, the commonly cited definition of neoplasm (tumour), formulated by the British oncologist R.A. Willis, says: “A neoplasm is an abnormal mass of tissue, the growth of which exceeds and is uncoordinated with that of the normal tissues, and persists in the same excessive manner after cessation of the stimulus which evoked the change” (*3*).

4.1.2. Benign and malignant tumours

Tumours are divided into benign and malignant (malignancies or cancers). Benign tumours, in general, remain well differentiated and are characterized by slow growth (*4*). Benign neoplasms may be harmful e.g. by local pressure or obstruction, however, they grow locally, do not invade surrounding tissue and do not spread to distal sites of the body. In contrast, cancers infiltrate the underlying mesenchyme and form metastases in other organs. Furthermore, malignancies range from well differentiated to anaplastic (lacking differentiation) and their growth rate can be slow or rapid. Cancer typically arises from epithelium (carcinomas), however, it may also

originate from mesenchyme (sarcoma), hematopietic cells (leukemias), lymphoid tissue (lymphomas) or from the neural system (*2, 4*).

4.1.3. Carcinogenesis

The major groups of genes involved in cancer are oncogenes (activated protooncogenes) which stimulate cell proliferation, tumour suppressor genes which inhibit proliferation, as well as genes that regulate programmed cell death (apoptosis). A separate class of genes, whose altered expression influences cancer development, are genes involved in DNA damage repair. This group contributes to the altered expression of oncogenes, tumour supressors and apoptosis regulators by affecting the organism ability to repair nonlethal DNA damage and therefore predisposing to DNA mutations (*4*). Furthermore an emerging group of carcinogenesis regulators are epigenetic modifiers of DNA and histones, chromatin remodelers and noncoding RNAs (*5-10*).

Stages of carcinogenesis encompass tumour initiation, promotion and progression. During the initiation phase a normal cell acquires first irreversible alteration in the expression of an oncogene or a tumour suppressor as a result of DNA mutation (*11*). Such alternations in the genome occur mainly somatically. Rarely, however, a mutated gene can be inherited as it is the case in early childhood retinoblastoma (cancer of retina) with congenital mutational loss of one of the two *Retinoblastoma* (*Rb*) tumour suppressor loci. Inheritance of one dysfunctional allele greatly increases the chance of developing malignancy upon somatic loss of its other functional copy (*12-14*). Of note, recent studies have drawn more attention towards the role of epigenetic silencing of tumour suppressors in cancer initiation (*11*).

The promotion stage results in propagation of initiated clones, increasing the number of affected cells and thereby also the chance that one of them acquires further alterations in gene expression and undergoes malignant transformation (*11*). Tumour promoting agents/processes stimulate the proliferation of preneoplastic cells or inhibit their apoptosis. A classical well studied example of a tumour promoter is 12-O-tetradecanoylphorbol-13-acetate (TPA) which activates the protein kinase C (PKC) pathway by intercalating to the cell membrane and mimicking diacylglycerol (*15*). The cancer promoting effect of phorbol ester results from downstream activation of the transcription factors activator protein (AP) - 1 and nuclear factor-kappa B (NF-κB) (*16-19*). Among clonally expanding cells, acquisition of further alterations leads over

time to the development of a malignant phenotype and entry into the progression phase (*11*).

4.1.4. Hallmarks of cancer

The characteristic traits of cancer cells (or cancer) encompass sustained proliferative signalling, avoidance of growth suppression, resistance to proapoptotic signalling, limitless replicative potential, promoting angiogenesis, invasion of surrounding tissues, formation of distal metastases and evasion of immunosurveillance. Individual cancer acquires those hallmarks at different stages and via distinct mechanisms. However, there are two superior characteristics of cancer development, which enable all of the above mentioned other alterations, and those are genomic instability and the presence of inflammatory component in the tumour microenvironment (*20*).

Improper genome fidelity maintenance results from dysfunction of DNA damage detectors and activators of the repair systems, components of DNA repair machineries or proteins detoxifying mutagens before the DNA damage occurs. Furthemore, compromised surveillance systems, which in normal conditions direct genetically damaged cells to senescence or apoptosis, with a central role of “guardian of the genome” TP53 (p53), are also responsible for genomic instability. Increased rate of mutation acquisition gives rise to development of other cancer hallmarks (*20*). Genomic instability is characteristic for almost all cancers, including malignancies associated with hereditary conditions and sporadic tumours. Consistent with the “mutator hypothesis” (on occurrence of a genomic instability at early stage of carcinogenesis, which in turn increases mutation rate and therefore cancer development), there are several types of cancer resulting from inherited mutations in DNA repair mechanisms. For instance in the hereditary non-polyposis colon cancer (HNPCC), also known as Lynch syndrome, mutations of DNA mismatch repair genes lead to alterations in the number of short repeated nucleotide sequences, called microsatellite instability (MSI or MIN). As a consequence, frame-shift mutations in other genes occur. On the other hand, the main form of genomic instability in sporadic cancers, chromosomal instability (CIN), is proposed to be mediated by oncogen-induced DNA replication stress (stalling and collapse of the replication forks), resulting in induction of DNA double-strand breaks (DSBs). Furthermore, under these circumstances, the cells with loss of tumour supressor p53, which is a main regulator of growth arrest, senescence or apoptosis in response to DNA

damage, or ataxia telangiectasia mutated (ATM), which encodes a kinase activating p53 upon DNA damage, gain a further selection advantage in growing tumours (*21, 22*).

The other “superior” hallmark of cancer is ongoing inflammation. It has long been known that solid tumours are infiltrated by inflammatory cells owing Rudolf Virchow`s first observations of this phenomenon already in the 19th century (*23*). Inflammatory processes may result from cancer development (and e.g. oncogenic activation) or precede the development of a tumour (*24*). Malignant microenvironment contains an inflammatory component whose presence is usually associated with worse, but in some cases with better cancer patient prognosis, suggesting different priming/polarization of inflammation in cancer development (*25-30*).

As introduced above, cancers possess the abilities to evade immune surveillance, which allows tumour to coexist with the host immune system. One of further mechanisms of how cancers avoid recognition by the host`s immunity is loss or downregulation of major histocompatibility complex (MHC) class I. Decreased expression of MHC class I renders cancer cells invisible for the cytotoxic T lymphocytes (*31*). Furthermore, increased release of lactate in the tumour microenvironment inhibits the action of natural killer (NK) cells which represent another important players in the host`s tumour surveillance. Elevated concentration of lactate, which is associated with high glycolytic activity of cancer, directly attenuates the cytolytic function of NK cells as well as recruits myeloid-derived suppressor cells (MDSC) which further inhibit NK cells (*32*).

Well established features of cancer are sustained proliferative (associated with activation of oncogenes) and dysfunctional growth limiting signalling (related to loss of tumour suppressor genes). Increased proliferation rate in cancer can result from an autocrine/paracrine stimulation of the cancerous cell with overexpressed, self-produced or tumour-associated stroma cell-derived growth factors. Alternatively, elevation of the expression levels of a receptor recognizing growth factor available in a limited concentration in the tumour microenvironment can give a growth advantage to cancer cells. Similarly, mutational change in the structure of such receptor or the downstream components of its signalling pathway, resulting in their constitutive activation may be a mechanism of increasing proliferation rate in cancer. On the other hand, loss of the negative feedback upon stimulation of a growth factor

pathway also gives rise to excessive cell division (*20*). For instance, insulin growth factors (IGFs), which signal via phosphoinositide 3-kinase (PI_3K), are commonly overexpressed, whereas negative regulators of this pathway are often lost in cancer cells (e.g. loss of PTEN tumour suppressor) (*33, 34*). The other extensively studied examples of tumour suppressors associated with common human malignancies are RB (retinoblastoma) and TP53 (or p53) proteins. RB functions mainly via inhibition of the E2F transcription factor family, therefore preventing entry into S-phase of the cell cycle. Phosphorylation of RB by cyclin-dependent kinases (CDKs) leads to its inactivation, release of E2F and cell cycle progression. However, the tumour suppressor role of RB spans beyond interaction with E2F transcription factors. Retinoblastoma participates in the regulation of cell cycle arrest by interaction with S-phase kinase-associated protein 2 (Skp2) and/or anaphase promoting complex (APC) to stabilize CDK inhibitor p27kip1 (*35*). In turn, p53 responds to cellular stresses and abnormalities, and therefore is required for prevention of cell cycle progression upon recognition of cellular alterations including DNA damage, incorrect mitotic spindle assembly, activation of oncogenes, oxidative stress or insufficient levels of nucleotides (*36-43*). Activated p53 regulates mainly expression of the genes responsible for growth arrest, DNA repair, senescence and/or apoptosis (*44*).

Another well known property of cancer cells is the ability to avoid apoptosis. Among the regulators of programmed cell death are two groups. The first responses to intracellular stresses including DNA damage, cytokine deprivation and oncogene activation, whereas the other receives and processes the extracellular signals such as Fas ligand (FasL), TNF-related apoptosis-inducing ligand (TRAIL) and tumour necrosis factor α (TNF-α), which activate their cognate death receptors. The ultimate event leading to cell disassembly is the activation of caspase 9 and 8 in response to activation of intra- and extracellular pathways, respectively (*45*).

The intrinsic (or mitochondrial) pathway triggering apoptosis has been especially widely implicated in cancer pathogenesis. The execution of apoptosis in the intrinsic pathway depends mainly on the balance between pro- and antiapoptotic protein subfamilies of the BCL-2 family. BCL-2, BCL-X and BCL-W are examples of antiapoptotic subfamily members which bind and sequester proapoptotic regulators. Bax and Bak belong to proapoptotic BCL-2 molecular players permeabilizing the outer mitochondrial membrane. Another group of proapoptotic regulators of the

intrinsic apoptosis pathway are “BH3-only proteins” (e.g. p53 targets Puma and Noxa) which share the BCL-2 Homology region 3 (BH3) with BCL-2 family members. “BH3-only proteins” serve as specific damage sensors and direct antagonists of the antiapoptotic regulators. In turn, Bax and Bak seem to be activated following two possible models. In the direct model, activation of the proapoptotic proteins is mediated by binding “BH3-only proteins”, whereas in the indirect model, “BH3-only proteins” displace proapoptotic BCL-2 members from their antiapoptotic relatives. Activation of the Bax and Bak results in the release of cytochrom c, followed by the downstream recruitment of the cascade of caspases which perform cellular disassembly (*46*). The most common mechanisms of avoidance of apoptosis in cancer is loss of p53 tumour suppressor, increased expression of antiapoptotic regulators and/or survival factors or decreased expression of proapoptotic mediators (*20*).

Acquisition of limitless proliferative potential by cancer cells is another characteristic of tumour progression. Normal cells have limited number of cell cycles that can be accomplished before they enter replicative senescent due to progressive telomeric DNA truncation after each replication of the genome. Cancer cells preserve the activity of telomerase (the enzyme responsible for elongation of telomeres) which enables unlimited number of divisions (*47*).

Another feature of cancer which is crucial for tumour growth and metastasis is formation of new blood vessels. As the tumour diameter reaches up to 1-2 mm, hypoxia triggers an “angiogenic switch” to allow restoring blood supply, therefore oxygen and nutrient delivery (*48*). Angiogenesis is a process in which new blood vessels in hypoxic tumours are formed from existing established vasculature, associated with induction of endothelial cell proliferation and migration. The prototypical mediator of angiogenesis is vascular endothelial growth factor (VEGF). Its regulation in hypoxia and association with increased capillary density in human cancer samples was shown already over 20 years ago (*49*). Shortly afterwards, it became clear that hypoxia activates hypoxia inducible factors (HIFs), subsequently increasing the expression of VEGF (*50*). Angiogenesis is also closely related to inflammatory processes. Experiments employing stable knock-down of HIF-1α (oxygen-sensitive subunit of HIF-1) in a model of colon cancer xenograft growth in mice indicated sustained angiogenesis in the absence of functional HIF pathway. The

induction of proangiogenic interleukin 8 (IL-8) expression was shown to promote angiogenesis in HIF-1α knock-down-derived tumours. Hypoxic HIF-1α knock-down cells displayed upregulation of IL-8 as a result of activation of the major regulator of inflammatory responses nuclear factor – kappa B (NF-κB) (*51*). Of further importance for the angiogenic processes in cancer is the recruitment of myeloid cells to tumour microenvironment (*52*). Strong support for this notion are findings on the positive correlation between the number of tumor-associated macrophages (TAMs) and microvascular density observed in human cancers (*53-55*). Furthermore, clodronate liposomes-mediated depletion of monocytes resulted in decreased angiogenesis in a model of Lewis lung carcinoma xenograft growth in mice (*56*). TAMs are known to be a potent source of multiple angiogenesis stimulating factors including VEGF, IL-8, platelet derived growth factor β (PDGFβ) and matrix metalloproteinases (MMPs) (*52*).

As mentioned above, cancer cells acquire the ability to invade surrounding tissue and form distal metastases. The model of cancer cell metastasis embraces: local invasion of surrounding tissue, invasion of the blood or lymphatic vessels (intravasation), survival in the bloodstream, extravasation at the distal site, survival at that site and its colonization (formation of the macroscopic secondary tumour) (*57*). The key adhesion molecule involved in preserving the integrity of epithelial tissue is E-cadherin. Decreased expression of E-cadherin is a well established event in epithelial-to-mesenchymal transition (EMT), an initial step in metastatic process cascade (*58*). Loss of E-cadherin expression occurs frequently in human cancers and is associated with disease progression (*59-61*).

4.2. Hypoxia

4.2.1. Introduction to hypoxia and its assessment in tumours

Hypoxia, defined as insufficient oxygen supply, elicits adaptive changes, promotes survival or leads to apoptosis of affected cells. However, oxygen availability has been shown to be involved in the regulation of physiological conditions such as embryonic development, inflammation, wound healing and adaptation to high altitude, as well as pathological conditions including ischemic diseases and cancer (*62*).

Physiologically normal values of tissue oxygen partial pressure (normoxic conditions) range from 14.4 to 64.8 mm Hg. In some cases, however, e.g. in the thymus, kidney medulla and bone marrow these values drop even below 7.2 mm Hg due to unusual blood vessel networks at these sites (*63*). It has been shown in all human malignant tumours studied that the level of oxygenation is lower in cancerous tissue compared to the median value of its tissue of origin (*64*). Hypoxia is associated with poorer prognosis in patients with different tumours including best studied malignancies of cervix, head and neck and soft tissue sarcoma (*65-67*). To obtain this knowledge direct oxygen-sensitive microsensor electrodes (Eppendorf pO_2 histogrphy) were applied in cancer patients. Nevertheless, this method has its disadvantages such as invasiveness and limitation of the access of a needle probe only to certain tumours (*68*). Polarographic sensors have further drawback, oxygen is being consumed during the measurements, and this precludes their use for measuring the oxygenation over time (*69*). Successors of polarographic electrodes became fluorescence-based fiber-optic probes, sensors which are usually made out of fluorescent dye ruthenium chloride held in a polymer matrix. The fluorescence light emission by those probes is quenched in an oxygen-dependent manner and remains inversely proportional to oxygen partial pressure. An important advantage of optical sensors over polarographic probes is no oxygen consumption during the measurements (*69*).

For the detection of insufficient oxygen availability in tumours several endogenous hypoxia-induced proteins e.g. hypoxia inducible factor-1α (HIF-1α) and its target genes carbonic anhydrase 9 (CA-IX) and glucose transporter 1 (GLUT-1), can be

applied in tissue sample analysis. However, the expression of HIF-1α, CA-IX and GLUT-1 has sometimes been reported to show a pattern which clearly does not correlate to the direct tumour oxygen measurement results. There are several possible explanations for that including suboptimal immunohistochemistry protocols and the influence of tumour microenvironment on expression of those markers (*68*). For example, it has been shown that HIF-1α stabilization can be a result of acidosis, whereas glucose deprivation may abrogate hypoxic CA-IX induction or stimulate induction of GLUT-1 (*70-72*). In addition, overexpression of HIF and its target genes may occur due to oncogene/tumour supressor pathways dysfunction (*68*).

Among hypoxia imaging techniques, exogenous markers e.g. 2-nitroimidazole derivatives such as 1-[(2-hydroxy-3-piperidinyl) propyl]-2-nitroimidazole hydrochloride (pimonidazole) or 2-(2-nitro-1H-imidazol-1-yl)-N-(2,2,3,3,3-pentafluoropropyl) acetamide (EF5) can also be used in humans upon their injection preceding tumour biopsy/resection (*73-76*). Those compounds have been shown to be reduced and strongly interact with cellular macromolecules when the oxygen partial pressure drops below 10 mm Hg. Formation of such adducts in hypoxic conditions may be detected by specific antibodies (*76*). However, oxygen-independent staining of pimonidazole adducts was also reported, suggesting that hypoxia-independent effects may influence the results obtained with exogenous hypoxia markers (*69*).

Non-invasive *in vivo* tumour hypoxia assessment techniques include imaging with positron emission tomography (PET) and magnetic resonance imaging (MRI). Radiolabelled 2-nitroimidazoles such as [^{18}F] Fluoromisonidazole (FMISO), [^{18}F] Fluoroazomycin-arabinose (FAZA) and [^{18}F] EF5, and other oxygen-sensitive probes e.g. [^{60}Cu] or [^{64}Cu] Copper(II)-diacetyl-bis(N4-methylthiosemicarbazone) (Cu-ATSM) have been developed and applied for hypoxia imaging by PET in preclinical and clinical studies (*77*). MRI techniques, which do not directly measure oxygenation, provide indirect information on hypoxia in tumours. This estimation is possible by measuring the fluctuations of magnetic resonance, which reflects the concentration of deoxyhemoglobin, vascular blood flow, vascular blood volume, or the presence of oxygen in tissue. In particular, blood oxygenation level-dependent (BOLD) MRI relies on the measurement of ratio of erythrocyte content of deoxyhaemoglobin (having paramagnetic properties) to oxyhaemoglobin (which is diamagnetic) (*78*).

Although numerous approaches to evaluate hypoxia in tumours have been developed none of them has found widespread clinical application yet.

4.2.2. Sensing and response to hypoxia on the systemic and cellular level

Neurons within medulla oblongata are responsible for producing rhythmic signals to stimulate respiratory muscles in a manner which does not depend on awareness. The exact site where neurons coordinating respiratory rhythm in normal conditions are located, so called central pattern generator (CPG), remains unknown. However, multiple neurons located in medulla, pons and other brainstem regions stimulate specific phases of the respiratory cycle (respiratory-related neurons, RRNs) (*79*).

The most important tonic drive signals of CPG come from central and peripheral chemoreceptors which monitor the partial pressures of O_2 and CO_2, as well as pH. The peripheral chemoreceptors are situated in the carotid and aortic bodies. Both types are sensitive mainly to drop in arterial blood P_{O_2}. Of note, an increase in blood P_{CO_2} as well as lowering of pH makes them more sensitive to decreased oxygen levels. Central chemoreceptors are located on the “brain” side of the blood-brain barrier. Those receptors sense the increase in arterial P_{CO_2} and a decrease in cerebrospinal fluid pH, although the latter more slowly. Peripheral and central chemoreceptors deliver the tonic drive to CPG in parallel to their function in feedback regulation of the respiratory output (depth and frequency of ventilation) to stabilize blood values with regard to P_{O_2}, P_{CO_2} and pH. Decreased P_{O_2}, increased P_{CO_2} or lowered pH cause an increase in ventilation, aiming at acute raising blood P_{O_2}, decreasing P_{CO_2} and elevating pH, to restore their correct values (*79*).

Kidney is another organ responsible for monitoring systemic arterial blood oxygenation. Decreased blood oxygen content is detected by renal erythropoietin (EPO)-producing and oxygen sensing (REPOS) cells located along the corticomedullary oxygen gradient in the juxtamedullary cortex. Decreased oxygenation of the REPOSs leads to their proliferation and production of EPO. Next, endocrine secretion of EPO stimulates the erythropoiesis in bone marrow, resulting in counteracting the decreased oxygen blood content by the increase in blood oxygen transport capacity (*80*).

On the cellular level the main players involved in response to hypoxia in higher metazoans are HIF-1, HIF-2 and HIF-3, composed of the oxygen-dependent subunit HIF-1α, -2α or -3α, respectively, and their constitutively expressed interaction partner aryl hydrocarbon receptor nuclear translocator - ARNT (or HIFβ) (*81*). The HIF system was discovered already over 30 years ago, in the context of binding to a 18 bp element in the 3` enhancer of *EPO* gene by a nuclear factor induced in hypoxia in Hep3B cells (*82*). HIF-1α is ubiquitously expressed, whereas HIF-2α (also known as endothelial PAS domain-containing protein 1, EPAS1), which is a close relative of HIF-1α (both proteins share 48% of sequence homology), is expressed in a cell type-specific manner. HIF-2α expression is restricted to endothelium, stromal cells of brain, kidney and pancreas, parenchymal and stromal cells of the heart, as well as in parenchyma of liver and intestine (*83-85*). HIF-3α is less well characterized. However, it has been shown to be expressed in humans in at least seven splice variants, and appears to modulate the HIF-1α- and HIF-2α-mediated transcriptional response in hypoxia (*86*).

HIF-1α and HIF-2α contain several distinct functional domains including: basic helix-loop-helix (bHLH) and PAS (which are responsible for dimerization and DNA binding), oxygen-dependent degradation (ODD) domain (required for degradation by the ubiquitin-proteasome pathway) and two transactivation domains (NTAD and CTAD) (*63*).

In normal oxygen conditions, prolyl-4-hydroxylase domain-1 (PHD1), PHD2 and PHD3 (also known as EGLN2, 1 and 3, respectively) hydroxylate two proline residues (402 and 564, and 405 and 531, in human HIF-1α and HIF-2α, respectively) in the ODD domain of HIF-α, marking it for interaction with ubiquitin ligase E3 von Hippel-Lindau (VHL) tumour suppressor, and subsequent proteasomal degradation (*87-91*). PHD2 knock-out in mice leads to embryonic lethality between days 12.5 and 14.5 due to severe placenta and heart malformation (*92*). Interestingly, knock-outs of PHD1 or PHD3 do not lead to any apparent phenotype, however, the outcome of a double PHD1 and PHD3 knock-out is moderate erythrocytosis (*92, 93*). Of note, a fourth prolyl-4-hydroxylase domain protein, endoplasmatic reticulum transmembrane PHD4 was shown to hydroxylate HIF-α *in vitro* (*94*). PHD4 knock-out phenotype in

mice is characterized by mild kidney defect with minor late-onset proteinuria, and eye abnormalities observed in aging mice (*95*).

Hydroxylation of the asparaginyl residues Asn803 and Asn851, in human HIF-1α and HIF-2α, respectively, by the factor inhibiting HIF (FIH), prevents binding of the transcriptional coactivators p300/CBP, and therefore inhibits HIF activity in normoxia (*96*). Mice lacking FIH largely do not differ from the wild type animals regarding classical aspects of HIF functions, e.g. vascularization, erythropoiesis or development, however, they display lower body weight, increased rate of metabolism and hyperventilation, as well as are resistant to high-fat-diet-induced gain of weigh and hepatic steatosis (*97*).

The activity of PHD and FIH enzymes is inhibited in hypoxia, therefore HIF-α subunits can rapidly accumulate and interact with HIFβ and p300/CBP to activate the transcription of target genes (*63*). An overview of the oxygen-dependent regulation of HIF-1 is shown in Figure 1. The importance of HIFs is underscored by the HIF-1α knock-out mouse embryo phenotype of severe cardiac and vascular malformations and lethality by day E10.5 (*98*). Similarly, lack of HIF-1β leads to a defective vascularization of the yolk sack and/or the embryonic part of the placenta, and death of the embryo by the E10.5 day (*99, 100*). In addition HIF-1β deficiency results in a decreased number of hematopoietic progenitors of the yolk sac (*101*). On the other hand, the results of global HIF-2α deletion remain model-dependent. In one study, knock-out embryos displayed severe vascular defects in the yolk sac, and died between days E9.5 and E12.5 (*102*). In another work, lack of HIF-2α resulted in fatal respiratory distress syndrome (RDS) in neonatal animals, which was associated with insufficient surfactant production due to decreased VEGF expression (*103*). Finally, a third group showed, that mice lacking HIF-2α died by day E16.5 of heart failure (*104*).

HIFs bind to the minimal consensus HIF binding site (HBS) CGTG within a hypoxia response element (HRE). However, based on the analysis of 107 known HBSs in 70 well characterized HIF target genes, it was found, that the closest adjacent nucleotides occur also with nonrandom frequency e.g. A is present in the -1 position and C in the +5 position, in more than 75% and over 40% of all known HBSs, respectively (*62*). Furthermore, Chromatin Immunoprecipitation-sequencing (ChIP-

seq) analysis of over 400 HBSs revealed further sequence preferences such as for T in the -2 position and CAC motif between +13 and +15 positions (*105*).

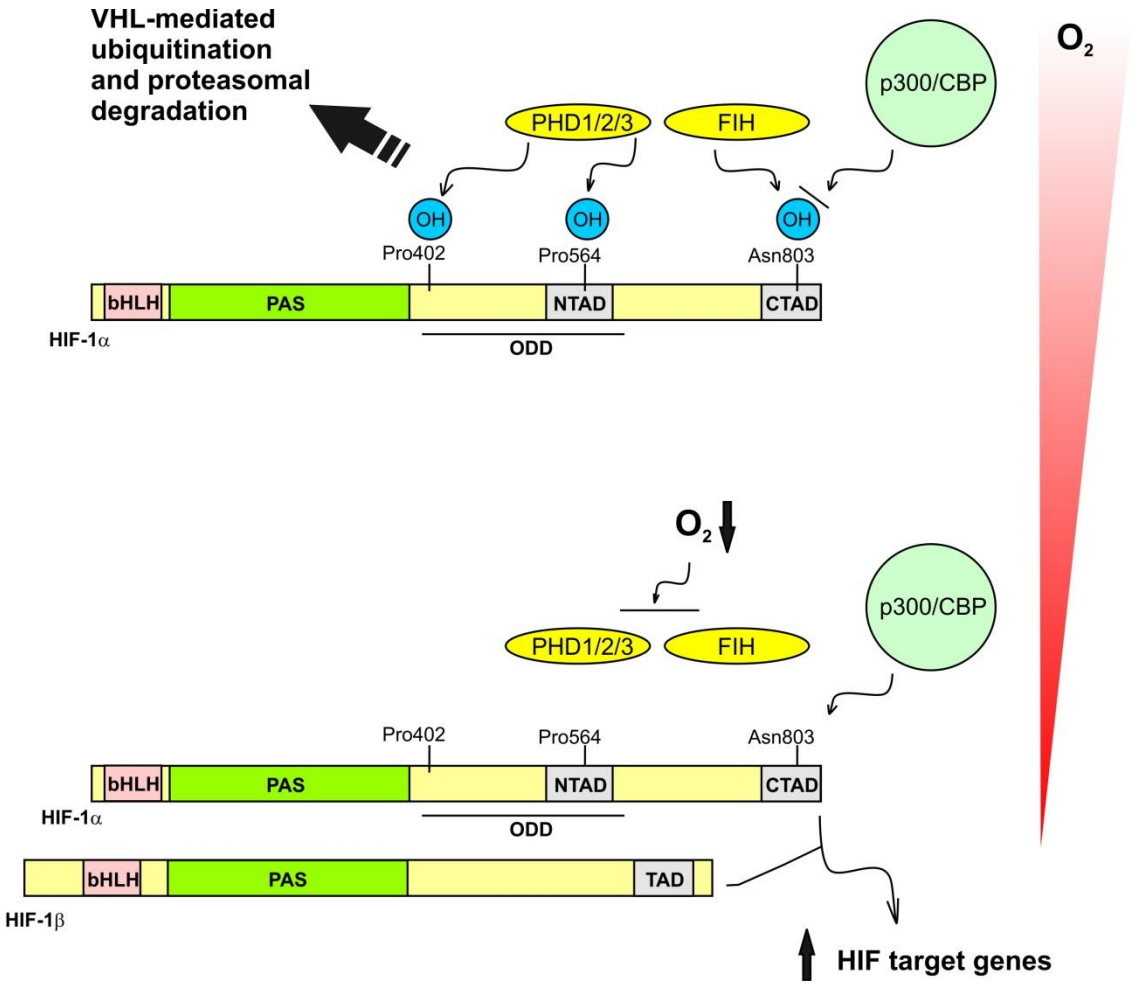

Figure 1 Oxygen-dependent regulation of HIF-1. In normal oxygen conditions PHD1/2/3 mediate Pro402 and 564 (in human) hydroxylation in ODD domain of HIF-1α, enabling its interaction with VHL and proteasomal degradation. On the other hand, FIH-mediated hydroxylation of Asn803 prevents HIF-1α from interacting with p300/CBP transcriptional co-activator. In the conditions of decreased oxygen partial pressure, PHD 1/2/3 and FIH enzymes are inhibited, therefore HIF-1α is stabilized, and binds HIF-1β and p300/CBP to transcriptionally regulate the expression of target genes (schematic organization of HIF subunits – modified from Schofield C.J., Ratcliffe P.J., 2004 (*106*)).

The HRE is defined as the minimal cis-regulatory element required for hypoxic induction of gene transcription. The presence of a HBS motif in a DNA sequence is mandatory but insufficient for hypoxia-mediated gene regulation. Binding of other transcription factors (not only hypoxia-inducible) leads to amplification of hypoxic response or confers tissue-specificity of hypoxia-targeted genes. For instance,

transcriptional activation of lactate dehydrogenase A (LDHA) requires ATF-1 and CREB-1 in addition to HIF-1, whereas AP-1 transcription factor cooperates with HIF-1 to activate the expression of vascular endothelial growth factor (VEGF) (*62*). Several tandemly arrayed HBS may also function as a complete HRE as it is the case for the transferrin (TF) gene, several glycolytic enzymes and glucose transporter-1 (GLUT-1) (*62*).

4.2.3. Hypoxia/HIF as powerful players in cancer

The presence of hypoxic cells in human tumours was described for the first time in 1955 by Thomlinson and Gray. All investigated by them tumours of more than 200 μm radius, which approximately corresponds to the tissue oxygen diffusion limit, displayed necrotic cores. This observation suggested that the necrotic cells belong to the region of no oxygen availability, and that the adjacent cells must be exposed to decreased oxygen conditions. Together with the preceding work by Gray, which showed that low oxygen availability limits cell death after exposure to ionizing radiation, these findings strongly implied, that the hypoxic cells in tumour compose the mayor obstacle in effective cancer radiotherapy (*107*).

A growing tumour is a dynamic structure with the cycles of local hypoxia and reoxygenation, while initially the distance to the nearest blood vessel increases resulting in hypoxia, and subsequently the blood flow is restored in the process of angiogenesis. Implications of periods of insufficient oxygen supply in cancer are manifold. As mentioned above one of the many important consequences of hypoxia is increased resistance to anticancer treatment. Interestingly, until 1972 there had been no explanation why multiple doses treatment is more efficient in tumour eradication than one single high dose of radiation. Ultimately, the work of Kallmann describing reoxygenation following irradiation of hypoxic tumours suggested, that the exposure to fractionated treatment may be beneficial due to normalization of oxygen conditions in initially hypoxic cancer sites (*108*).

Among underlying mechanisms of hypoxia-mediated anticancer therapy resistance is the oxygen-mediated fixation of the unpaired electrons of free radicals induced by radiation. Efficient killing of normoxic mammalian cells requires 2.5 - 3 times lower dose of radiation than the same procedure performed in decreased oxygen conditions. On the other hand, hypoxic cells are the most difficult to target with

anticancer drugs due to their remote location with regard to blood vessels and decreased proliferation rate (*64*).

Another aspect of hypoxia in cancer is the induction of profound molecular programs associated with expression of hypoxia inducible factors (HIFs), which influence the development and progression of a malignancy, as well as the treatment efficiency. HIF target genes are involved in glycolysis, cellular proliferation, survival or apoptosis, angiogenesis, invasion, metastasis and other processes, which will be described in more detail below.

90 years ago Otto Warburg found, that thin slices of tumours *ex vivo* were characterized by increased glucose uptake and lactate production even in aerobic conditions. This phenomenon is called Warburg effect or aerobic glycolysis. The cause of increased glycolysis rate in aerobic conditions in cancers is partially the dysregulated expression of oncogenes/tumour supressors, however, this effect is strongly fueled by hypoxia- or nonhypoxic pathways-mediated stabilization of HIF-1 (*109*). HIF targets are involved in regulation of glucose uptake (e.g. GLUT-1 and -3), glycolysis (e.g. lactate dehydrogenase A - LDHA, phosphoglycerate kinase 1 - PGK1, aldolase A and enolase 1), as well as inhibition of oxidative phosphorylation e.g. pyruvate dehydrogenase kinase 1 (PDK1) (*110-115*). Additionally, another well established HIF target gene CA-IX participates in normalization of the decreased intracellular pH (resulting from excessive lactate production) giving the cancer cells survival advantage in hypoxic and acidic microenvironment (*116, 117*).

As a master of adaptation to hypoxic conditions HIF increases local blood flow to compensate for decreased oxygen concentrations in the blood. An increase in nitric oxide (NO) which is a major regulator of vasodilation and product of inducible nitric oxide synthase, iNOS (also known as NOS2) leads to widening of vessels and thus increased blood perfusion of a hypoxic region (*118*). The product of another HIF-target gene cyclooxygenase-2 (COX-2), also known as prostaglandin-endoperoxide synthase 2 (PTGS2), leads to the synthesis of prostaglandin E_2 (PGE_2) which has similar regulatory effect on the vasculature like NO (*119, 120*).

HIF is also a major orchestrator of angiogenesis, a process in which new blood vessels are created via sprouting of existing ones. The examples of well known HIF target genes involved in this process are the main regulators of endothelial

recruitment and proliferation, vascular endothelial growth factor (VEGF) and its receptors VEGFR1/R2 (*50, 121, 122*). Angiopoietin 2 (Ang-2) and TEK receptor tyrosine kinase (also known as tunica interna endothelial cell kinase, Tie-2) represent another examples of angiogenesis regulatory proteins under the control of HIF (*85, 123*). Hypoxia-inducible Ang-2 is a natural antagonist of Ang-1, whose signalling via Tie-2 receptor mediates maturation and stabilization of the vessels. However, the action of Ang-2 destabilizes existing vessels and promotes their sprouting by disruption of the connection between endothelial and periendothelial cells, enhancing at the same time the effect of VEGF (*124*). Another HIF target PDGF-β stimulates recruitment of pericytes that are required for the stabilization of the new tumour vasculature (*125, 126*). Matrix metalloproteinases 2 and 9 (MMP2 and 9), which were found to be directly transcriptionally targeted by HIF, play an important role in tumour angiogenesis, enabling endothelial cells to migrate through surrounding tissue and invade the tumour mass, among other functions (*127-131*). Similarly, urokinase-type plasminogen activator receptor (uPAR), also known as plasminogen activator, urokinase receptor (PLAUR), which is a regulator of plasminogen activation system (mediating tissue proteolysis), was shown to be regulated in a HIF-dependent manner (*132*). uPAR was shown to be required for the degradation of extracellular matrix in front of the leading edge of migrating endothelial cell (*133*). Moreover, products of iNOS and COX-2 are also proangiogenic factors, which upregulate the expression of VEGF (*134-137*). Furthermore, hypoxia leads to HIF-dependent upregulation of interleukin 8 (IL-8), which is a known angiogenesis modulator involved in endothelium survival and proliferation, as well as secretion of MMP2 and MMP9, therefore contributing to formation of the new vessels (*138, 139*). It has also recently been shown, that HIF-1α induces the recruitment of different populations of bone marrow-derived $CD45^+$ myeloid cells (vascular modulators, which are not incorporated to the vasculature) as well as endothelial and pericyte precursor cells, which promote vasculature growth in glioblastoma. This action of HIF-1α is at least partially dependent on the expression of chemokine ligand CXCL12, also known as stromal cell-derived factor 1 (SDF-1) (*140*).

An important role of HIF in cancer is regulation of cell survival/ apoptosis and proliferation. For instance, insulin-like growth factor 2 (IGF-2) and transforming growth factor α (TGFα), which are implicated in cellular proliferation, are HIF-1-

regulated genes (*127, 141*). However, HIF-1 was shown to functionally counteract c-Myc thereby derepressing p21 (CDK-interacting protein 1, CIP1) and inhibiting cell cycle progression in colon carcinoma cells (*142*). In turn, HIF-2 promoted the transcriptional activity of c-Myc stimulating cell cycle progression in renal cell carcinoma (RCC), NIH3T3, HEK293 and embryonic epithelial cells (*143*). Of note, chronic hypoxia may result in acquisition of proapoptotic phenotype via BCL-2/adenovirus E1B 19 kDa interacting protein 3, BNIP3 (also known as NIP3) which is a proapoptotic protein induced in a HIF-dependent manner (*144, 145*). On the other hand, COX-2/prostaglandin E2 may play a role in cell survival and/or proliferation for instance by recruiting PI_3K/AKT, mitogen-activated protein kinase (MAPK), cyclic adenosine monophosphate/protein kinase A (cAMP/PKA) and epidermal growth factor receptor (EGFR) signalling pathways in colon cancer cells (*146*).

Among HIF target genes critically involved in carcinogenesis is lysyl oxidase (LOX), a gene required and sufficient to repress E-cadherin expression and promote epithelial-to-mesenchymal transition (EMT), which is necessary for cancer invasion and metastasis (*147, 148*). A related role has been shown for other genes regulated by HIF, snail family zinc finger 1, SNAI1 (also known as Snail) and zinc finger E-box binding homeobox 2, ZEB2 (also known as SIP1), both acting as repressors of E-cadherin expression (*149*). HIF target genes have further roles in the development of proinvasive and prometastatic phenotype. For instance, HIF-1α overexpression-mediated invasiveness of colon cancer cells was blocked by neutralization of uPAR (*127*). Expression of uPAR was also implicated in the (angio)invasiveness of pancreatic and liver cancer cells (*150*). Degradation of extracellular matrix elicited by MMP2 and 9 further contributes to HIF-mediated invasion and metastasis by facilitating breakdown of epithelial basal membrane and underlying tissue, as well as intravasation by tumour cells (*130, 151*). Other HIF target genes such as chemokine receptor CXCR4 and its ligand CXCL12/SDF1 have a well established function in stimulating proliferation and homing of cancer cells to distal sites of the body (*152-156*).

Hypoxia contributes also to the genomic instability in cancer cells. For instance, transcriptional repression of MSH2 and MSH6 (DNA mismatch repair system components) in hypoxia, is dependent on HIF-1-mediated displacement of activator c-Myc from binding to Sp1 transcription factor, and concurrent hypoxic attenuation of

p53 *MSH2* and *MSH6* promoter DNA binding (*157*). In turn, NO (product of iNOS) may induce DNA damage or inhibit DNA repair systems mediating mutagenesis (*158*).

The classical stimulus activating HIF-α is hypoxia leading to the inhibition of PHD/FIH enzymes (*63*). Hypoxia occurs in solid cancers which outgrew the oxygen diffusion limit and have not yet developed intrinsic vasculature or the newly formed vessels have improper/leaking architecture. However, constitutively high levels of HIFα are very common in cancers e.g. due to activation of oncogenes or loss of tumour suppressor genes (*159*).

Almost 70% of examined cancer types displayed upregulated expression of HIF-1α compared to their surrounding tissue. Among malignancies with dysregulated HIF-1α expression were common human cancers such as colon, breast, gastric, lung, skin, ovarian, pancreatic and prostate carcinoma (*160*).

Clinical investigations showed the association between increased HIF-1α expression and poor survival of patients with early-stage cervical cancer, breast cancer, oligodendroglioma, ovarian cancer, endometrial cancer and oropharyngeal squamous cell carcinoma (*161*). Similarly, in a cohort of over 700 colon cancer patients overexpression of HIF-1α was shown to be associated with higher colorectal cancer-specific mortality, whereas no correlation between HIF-2α expression status and patient survival was observed, implying that HIF-1α is the isoform responsible for a more aggressive phenotype in this type of malignancy (*162*). However, association between HIF-2α overexpression and increased patient mortality was established for non-small-cell lung cancer, head and neck carcinoma, neuroblastoma and astrocytoma. Intriguingly, non-small-cell lung and head and neck carcinomas which display upregulated expression of HIF-1α have better survival prognosis suggesting the possible opposing roles of HIFs in some types of malignancies (*161*).

A malignancy example of well understood differential role of HIFα subunits is clear cell RCC (ccRCC). In about 70% of sporadic ccRCC cases *VHL* tumour suppressor is lost which results in stabilization of HIFα subunits (*163*). However, HIF-1α and HIF-2α were shown to exert opposing effects on growth of RCC xenografts, with overexpression of HIF-1α attenuating and HIF-2α accelerating tumour growth (*164*).

It was further reported, that HIF-2 stimulates c-Myc transcriptional activity and therefore cellular proliferation, whereas HIF-1 counteracts this effect in RCC cells (*143*). Consistently, elevated activity of c-Myc and enhanced cell proliferation is associated with loss of *VHL* and stabilization of HIF-2α, but not with loss of *VHL* and expression of both HIF-1α and HIF-2α or the presence of intact *VHL,* in primary sporadic RCC tumours (*163*).

4.3. Inflammation

4.3.1. Inflammation as a mechanism of response to pathogenic insult

Inflammation is described by four basic characteristics: Lat. *calor* (heat), Lat. *dolor* (pain), Lat. *rubor* (redness) and Lat. *tumour* (swelling) recognized already by Celsus in the 1st century. Traits of inflammatory processes are associated with local vasodilatation, increased vascular permeability, increased blood flow and fluid leakage, as well as accumulation of inflammatory cells at the site of injury (*165*).

Great input into understanding of these processes had studies of Elie Metchnikoff which in 1882 performed an experiment on a transparent larvae of a starfish pierced with a rose thorn. Examination of the larvae on the next day revealed accumulation of ameboid cells covering the thorn which, according to him, aimed at engulfing and removing the thorn. Metchnikoff was the first to describe phagocytosis as a fundamental mechanism by which creatures belonging to the animal kingdom remove foreign and potentially dangerous invaders to their organisms (*166*).

The immune processes are divided into innate and adaptive responses. Basic differences between those two are the time of onset (innate response acts within minutes, adaptive requires 4-7 days to develop) and their specificity. Common molecular components of pathogens are recognized by innate response, whereas specific antigens of a particular invader are detected by adaptive response (*165*).

Most of the pathogens activate the innate response first. Tissue macrophages directly recognizing molecular markers on pathogens e.g. lipopolysaccharide (LPS), which is a component of the outer membrane of Gram-negative bacteria, get activated, engulf invaders and produce inflammatory mediators. Those mediators include cytokines (modifiers of the behavior of cells carrying a corresponding receptor) and chemokines (attractants for cells expressing a cognate receptor). Cytokines and chemokines released by activated macrophages initiate the inflammation. Also complement, a set of plasma proteins which upon recognition of pathogen interact with one another to opsonize (or coat) the invader, can trigger inflammatory processes. This occurs through enhancement of phagocytic function of cells expressing the receptors against complement coating a pathogen, as well as

through release of small pieces of some of its activated components which subsequently act as chemoattractants and activators of phagocytes (*165*).

The groups of leucocytes involved in innate immunity are granulocytes, macrophages, dendritic cells, mast cells and NK cells, whereas adaptive response relies on lymphocytes activation. Many infections are successfully eradicated by innate immunity, however, when the first line of response fails, adaptive immunity mechanisms are triggered (*165*).

4.3.2. Cells and their products involved in inflammatory response

The immune cells (white blood cells, leucocytes) originate from bone marrow and are produced from precursor cells common for all blood cellular components – the hematopoietic stem cells. Many of the leucocytes maturate also at the site of origin, however, some migrate to the thymus or peripheral tissues to accomplish their developmental processes. In the bone marrow, initially pluripotent stem cells differentiate to lineages which have more specialized and limited potential. Those are common lymphoid and common myeloid progenitors (*165*).

Common myeloid progenitor lineage specializes to the megakaryocyte/erythrocyte progenitor (from which platelets and red blood cells, respectively, develop) and granulocyte/macrophage progenitor (which gives rise to granulocytes, macrophages, dendritic cells and mast cells) (*165*).

Granulocytes (or polymorphonuclear, PMN cells) contain densely stained granules in their cytoplasm. Neutrophils are the most common cells among granulocytes. Their role relies on the phagocytosis of pathogens and further promotion of inflammatory response. Antibody coated parasites are in turn targeted by eosinophils. The role of the third kind of granulocytes, basophils, is not yet fully understood, although they seem to act similar to the mast cells (described below) (*165*).

Macrophages originate from their precursors (monocytes) circulating in blood and differentiating upon migration to the local tissues. Their most important role is to engulf the pathogen, kill it and release molecular mediators that help attract and activate other components of inflammatory response (including neutrophils and T lymphocytes, or T-cells). In contrast, dendritic cells (DC) are specialized to

phagocytise a pathogen, process it and present its antigens to T-cells (which do not recognize antigen unless presented by such antigen presenting cell, APC) (*165*).

Mast cells are another group of cells originating from the granulocyte/macrophage progenitor cells. Upon their activation, substances increasing vascular permeability, including histamine, are released. In addition to their well known role in driving allergic reaction, mast cells are also believed to participate in protecting the internal body surfaces against infections and play role in response to parasitic worms (*165*).

The common lymphoid progenitor differentiates to lymphocytes including B lymphocytes (B-cells), T lymhocytes (T-cells) and natural killer (NK) cells. Also some portion of dendritic cells develops from the lymphoid lineage (*165*).

B-cells originate and maturate in bone marrow, as opposed to T-cells which leave the bone marrow to continue the development in thymus. Small naive lymphocytes have no functional activity until they encounter the specific antigen as well as get the co-stimulatory signal from innate immunity response cells. Only after the double stimulation is accomplished, the antigen-specific cells start to proliferate and differentiate to their functional stage (*165*).

Activated B-cells differentiate to plasma cells which secrete antibodies against specific pathogen. Released antibodies neutralize viral particles and bacterial toxins, opsonize pathogens to enhance their phagocytosis, and activate complement (which as mentioned above acts as another coating mechanism, but also creates pores in, and therefore destroys some bacteria). The type of immune response associated with release of antibodies is known as humoral immunity (*165*).

T-cell mediated inflammatory processes are called cellular or cell-mediated immune responses of adaptive immunity. Naive T-cells leaving thymus carry mainly CD8 or CD4 antigen. Activation of CD8-positive cells results in differentiation to cytotoxic T-cells (Tc), whereas of CD4-positive in generation of a population of helper T-cells (mainly Th1 or Th2). The role of Tc lymphocytes is killing cells infected with intracellular pathogens or transformed cells. Th1 cells have a dual function. On one hand, Th1 cells control infections by intracellular pathogens, such as *Mycobacterium tuberculosis*, which may proliferate in a macrophage due to blocking the fusion of a phagosome with a lysosome. This infection can be eradicated through activation of such an infected macrophage by Th1 cell which recognized bacterial antigen

presented on the surface of a phagocyte. On the other hand, activated Th1 cells express co-stimulatory signals activating production of antibodies by B-cells. In contrast, Th2 cells are responsible exclusively for co-stimulation of B-cells (*165*). A distinct class of T-cells, regulatory (Treg) or suppressor (Ts) cells are negative modifiers of action of other lymphocytes providing maintenance of immunologic self-tolerance (*167*).

Other kind of lymphoid lineage-derived cells are the natural killer (NK) cells, which as opposed to B and T lymphocytes, do not express antigen-specific receptors and belong to the innate immune system. Circulating in the blood, similarly to T lymphocytes, those cells are able to recognize and kill intracellular pathogen-infected or transformed cells (*165*).

Lymphoid organs consist of tissues containing lymphocytes and non-lymphoid cells. Those organs are divided into central (primary) lymphoid organs and peripheral (secondary) lymphoid organs. Central organs (bone marrow and thymus) are places of generation/maturation of lymphocytes, whereas peripheral organs (lymph nodes, spleen, mucosal lymphoid tissues) are locations of antigen-specific activation and proliferation of lymphocytes, as well as control of their survival (limiting the number of circulating lymphocytes). Upon maturation, B- and T-cells enter the bloodstream to reach the peripheral lymphoid organs. Immature dendritic cells leave the bloodstream to enter tissues where they take up the pathogens. Rapid maturation after encountering the pathogen leads to expression of co-stimulatory molecules on the surface of dendritic cells, which translocate to the nearest lymph node, subsequently activating the T-cells (*165*).

4.3.3. Inflammation on the cellular level – NF-κB, the master regulator of inflammatory responses

Nuclear factor kappa light chain enhancer of activated B-cells (or nuclear factor-kappa B, NF-κB) was originally discovered over twenty five years ago as an inducer of kappa light chains in B lymphocytes (*168, 169*). It has been well established, that NF-κB is an ubiquitously expressed dimeric transcription factor induced in response to different stimuli.

NF-κB plays a central role in inflammatory responses by mediating immune cell function and promoting inflammation, which is associated with inducing expression of different cytokines, chemokines, and their receptors. Furthermore, NF-κB inhibits cell death in inflammatory conditions by stimulating the transcription of antiapoptotic genes (*170*). However, the response of NF-κB is context-dependent and in some cases may also elicit cell cycle arrest. In normal human epidermal cells, NF-κB and overexpression of oncogenic Ras trigger cell cycle arrest, whereas inhibition of NF-κB (by overexpression of IκBα) prevents oncogenic Ras-mediated induction of growth arrest and results in malignant transformation of normal cells (*171*).

NF-κB dimers continuously shuttle between the cytoplasm and nucleus but the balance is strongly shifted towards cytoplasmatic localization in unstimulated conditions, and the nucleus upon proinflammatory stimulation. In basal conditions, NF-κB remains associated with a member of a family of related inhibitory proteins (called IκBs) among which the prototypical and the most extensively studied is IκBα. IκBα masks the nuclear localization signal (NLS) of NF-κB as well as carries the nuclear export signal (NES), which ensure cytoplasmatic localization of NF-κB in complex with IκBα. Proinflammatory signalling leads to degradation of IκBα and liberation of NF-κB which then translocates to the nucleus and activates its target genes (*172*).

NF-κB acts as a homo- or heterodimer composed of REL homology domain (RHD) protein family members including p65/RelA, RelB, c-Rel, p105/p50 (NF-κB1) and p100/p52 (NF-κB2), in mammals. The p65/p50 binding partners represent the most abundant dimers being found in almost all cell types (*173*). However, the prevalence of different NF-κB dimers in response to proinflammatory stimulation depends on the cell type, stimulus and time after exposure (*170*). The overview of RHD proteins is shown in the Figure 2.

Notably, mice with deletion of p65/RelA display embryonic lethality at the stage E15-16 due to massive hepatic apoptosis (*174*). On the other hand, lack of p50 subunit expression does not affect the development of mice, but renders them defective in the functions of the immune system (*175*).

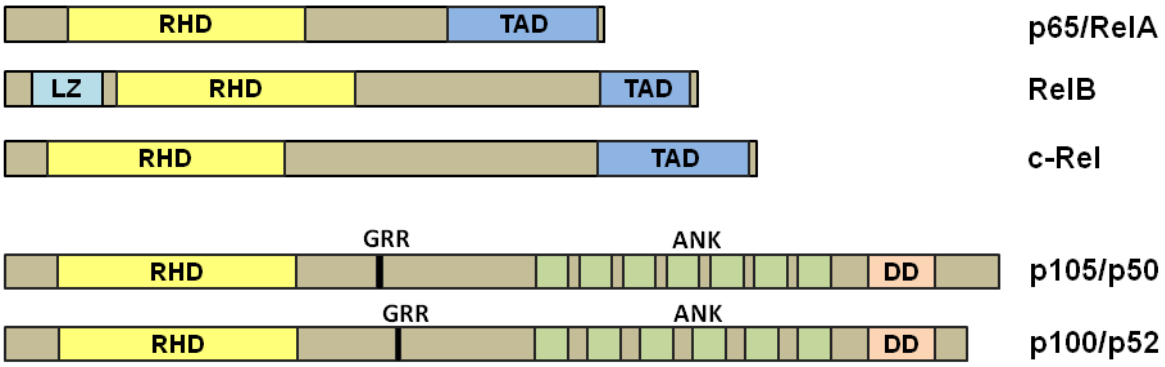

Figure 2. NF-κB subunits. The mammalian RHD protein family is composed of p65/RelA, RelB, c-Rel, p105/p50 (NF-κB1) and p100/p52 (NF-κB2) members. RHD – REL homology domain; TAD – transactivation domain; LZ – leucine zipper domain; GRR – glycine-rich region; ANK – ankyrin repeat domain; DD – death domain (modified from Oeckinghaus A., Ghosh S., 2009).

The RHD at the N-terminus of RHD family proteins is composed of approximately 300 amino acids, and mediates binding the inhibitory IκB proteins, dimerizartion of subunits, nuclear translocation and association to DNA at the consensus κB site 5´-GGGRNNYYCC-3´ sequence. Only the p65/RelA, RelB and c-Rel carry a transactivation domain (TAD), which is located at their C-termini and confers the transcription activation ability of NF-κB dimers (*173*).

RHD proteins p50 and p52 are produced by cleavage of the p105 and p100 precursor, respectively. Proper processing of p50 and p52 depends on glycine-rich region (GRR), which serves as termination signal for their proteosomal degradation. Both p105/p50 and p100/p52 are devoid of TAD, therefore those two subunits bound to DNA as homo- or heterodimers may act as suppressors of κB-dependent transcription. C-termini of the p105 and p100 precursors contain ankyrin repeats, which are characteristic for the IκB family, making them be also members of the below described IκB group of proteins (*173*).

IκB family members interact with RHD proteins via ankyrin repeats, and this interaction inhibits the nuclear translocation of NF-κB dimers. In the majority of unstimulated cells inactive NF-κB dimers are kept in the cytoplasm by typical IκB proteins (IκBα, IκBβ or IκBε) or precursor RHD proteins p105 and p100. Interestingly, atypical members of IκB family BCL-3 and IκBζ can be induced upon

proinflammatory stimulation and likely ensure the transcription activation properties of some NF-κB dimers (*173*). An overview of different IκBs is shown in the Figure 3.

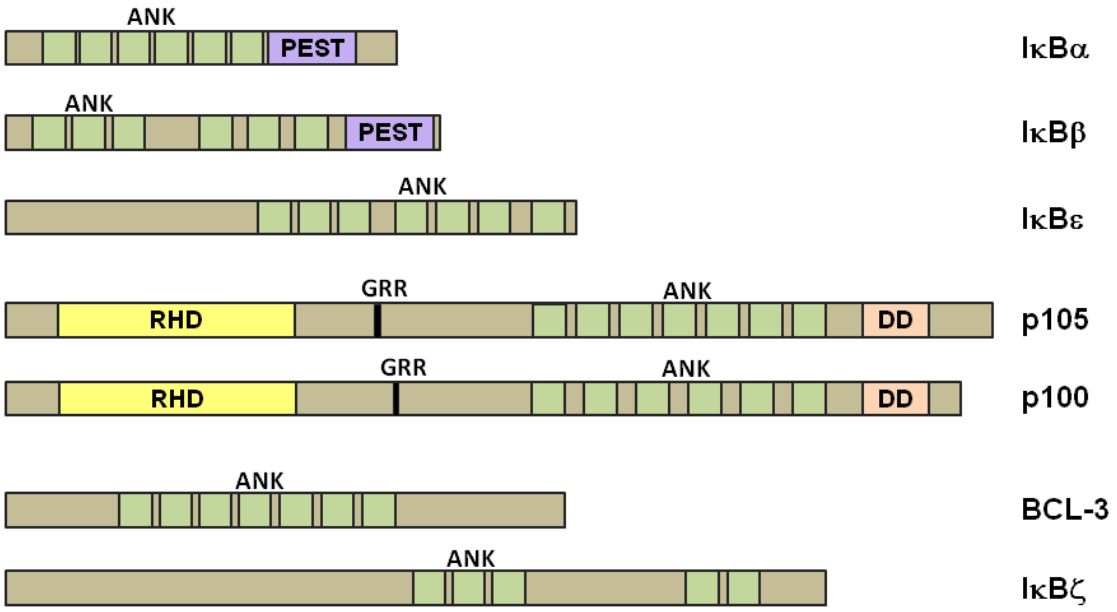

Figure 3. IκB proteins. IκB family represented by typical IκBs (IκBα, IκBβ and IκBε), precursor RHD proteins p105 and p100, as well as atypical members BCL-3 and IκBζ. ANK – ankyrin repeat domain; PEST - proline-, glutamic acid-, serine- and threonine-rich region; RHD – REL homology domain; GRR – glycine-rich region; DD – death (modified from Oeckinghaus A., Ghosh S., 2009).

IκBα and IκBβ bind mainly dimers containing p65/RelA or c-Rel (and IκBα inhibits mainly the most abundant p65/p50 heterodimer). On the other hand, p100 exclusively captures the RelB subunit. In turn, BCL-3 and IκBζ interact preferentially with dimers being combinations of p50 and p52 (*173*).

NF-κB activation modes are classified as canonical (classical) and noncanonical (alternative) pathways. Classical stimulation of NF-κB activity is elicited by interleukin 1 (IL-1), tumour necrosis factor α (TNF-α), bacterial products e.g. LPS, or activation of antigen receptors. Among noncanonical inducers of NF-κB are cluster of differentiation 40 ligand (CD40L), B cell activating factor of the TNF family (BAFF) and lymphotoxin-β (*172*).

The canonical mode of NF-κB stimulation embraces activation of the IκB kinase (IKK) complex and therefore downstream phosphorylation of the IκBs, mainly IκBα, followed by its ubiquitination and proteasomal degradation. IKK complex activated in canonical pathway consists of one copy of each kinase subunit IKKα and IKKβ, and

most probably two copies of regulatory IKKγ (also known as NF-κB essential modulator, NEMO). Degradation of IκBα in this pathway is mediated by its IKKβ-driven phosphorylation. This event leads to liberation of NF-κB (predominantly of the classical heterodimer p65/p50), followed by its nuclear translocation and activation of target genes. Among them is IkBα itself, resynthesis of which is a very important negative feedback mechanism controlling the NF-κB pathway activity (*172*).

In contrast, noncanonical activation of NF-κB involves activation of the NF-κB-inducing kinase (NIK) which phosphorylates and activates the IKKα kinase. IKKα-mediated downstream phosphorylation of the p100 (NF-κB2) subunit in complex with RelB, leads to its proteolytic cleavage to p52 and therefore activation of dimers (*172*).

Furthermore, atypical activation of NF-κB may occur via IKK-independent mechanisms. For instance, UV radiation exposure or expression of the Her2/neu oncogene leads to phosphorylation and degradation of IκBα which is mediated by casein kinase II (CK2) and independent of IKK (*176, 177*).

4.3.4. Toll-like receptor signalling

The involvement of Toll receptor in the activation of immune responses in a *Drosophila* cell line was shown first in 1995 by Rosetto and colleagues (*178*). Shortly afterwards, a human homologue of Toll gene (hToll) was described (*179*). Later studies revealed the presence of 10 functional members of Toll-like receptors (TLRs) in humans and 12 in mice. TLR1-9 are conserved in mice and man, TLR10 is dysfunctional in mouse due to retrovirus insertion, whereas TLR11-13 are not present in the human genome. Those receptors can be divided into a group expressed on the cell surface (TLR1, TLR2, TLR4, TLR5 and TLR11), and a group located in intracellular vesicles such as endoplasmic reticulum, endosomes, lysosomes and endolysosomes (TLR3, TLR7, TLR8 and TLR9) (*180*).

TLRs recognize a whole spectrum of pathogen-associated molecular patterns (PAMPs). For instance, LPS of Gram-negative bacteria is a ligand of TLR4 (hToll). TLR2, acting as a dimer with TLR1, is responsible for recognition of triacylated lipopeptides from Gram-negative bacteria and mycoplasma. The heterodimer composed of TLR2 and TLR6 is responsible for signalling diacylated lipopeptides from Gram-positive bacteria and mycoplasma. TLR5 detects bacterial flagellin (a

component of bacterial flagella). Mouse TLR11, detects profilin-like protein of a parasite *Toxoplasma gondi* and some components of uropathogenic bacteria. TLR3 recognizes viral double-stranded RNA (dsRNA) and certain small interfering RNAs (siRNAs). TLR7 responds to single-stranded RNA (ssRNA) derived from RNA viruses such as HIV-1 and influenza A virus, as well as certain siRNAs. Similar function of viral ssRNA recognition has its closest relative TLR8. TLR9 is responsible for sensing unmetylated CpG motifs in bacterial and viral DNA (*180*).

TLRs are composed of an extracellular leucine-rich repeat (LRR) domain (governing the recognition of pathogens) and a cytoplasmatic Toll/interleukin-1 receptor (TIR) domain (necessary for initiation of intracellular signalling) (*181*). Activation of the TLR signalling results in the upregulation of inflammatory cytokines and chemokines due to activation of NF-κB transcription factor and MAPKs (which includes downstream activation of AP-1). In addition, type I interferon (IFN) response is activated in the case of TLR3, -4, -7/8 and -9 receptor stimulation (*180*).

After binding the ligand, TLRs interact with the TIR-domain-containing cytosolic adapters and this event initiates the signalling cascade. Adapter proteins involved in TLR signalling include myeloid differentiation primary response protein 88 (MyD88), TIR domain-containing adapter protein (TIRAP), also known as MyD88-adapter-like (MAL), TIR domain-containing adapter inducing interferon β (TRIF) and TRIF-related adapter molecule (TRAM). Almost all TLRs (except TLR3) utilize MyD88 adapter and TLR4 recruits all four of them (*180*). An overview of the basic principles of TLR signalling is shown in Figure 4.

In MyD88-dependent pathways, the interaction between TLR and this adapter recruits interleukin-1 receptor associated kinase 1 and 4 (IRAK1 and 4) (*180*). First, IRAK4 becomes activated upon association with MyD88 adapter. Next, IRAK4 phosphorylates IRAK1 and this event induces second (autocatalytic) phosphorylation and further activation of IRAK1. Hyperphosphorylated IRAK1 interacts with tumour necrosis factor receptor-associated factor 6 (TRAF6). Activated TRAF6 mediates ubiquitination-dependent downstream signalling leading to NF-κB and MAPK activation (*182*).

MyD88-independent responses upon stimulation of TLR3 or TLR4 signalling proceed via TRIF adapter. It was shown by Chang and colleagues, that pellino E3 ubiquitin

protein ligase 1 (Peli1) governs the ubiquitination of receptor-interacting protein kinase 1 (RIP-1), and is involved in cytokine production in response to stimulation of TLR3 and TLR4 receptors (*183*). RIP-1 was previously shown to mediate the TRIF-specific response of NF-κB to TLR3 and TLR4 stimulation (*184*). Furthermore, it was also demonstrated that the intracellular death domain (DD)-containing adaptor, TNFR1-associated DD protein (TRADD), which can recruit receptor-interacting protein kinase 1 (RIP1) upon TNF-α stimulation, associates with receptor and mediates the TRIF-dependent NF–κB induction also in TLR4 and TLR3 signalling (*185*). On the other hand, TRIF was also suggested to recruit TRAF6 and therefore downstream complex TAB-TAK1 upon TLR3 signalling activation (*186*).

Activation of TRIF-adapter-mediated signalling culminates at induction of inflammatory cytokines and chemokines, but also secretion of type I interferon, in particular interferon-β. Transcription of type I interferon is controlled by several factors such as NF-κB, ATF2/cJun (AP-1) and interferon regulatory factor 3 or 7 (IRF3 or 7). IRF3 and IRF7 are major controllers of type I IFN response. Similarly to NF-κB, in unstimulated conditions IRF3 and IRF7 remain inactive in the cytoplasm (*187*). Phosphorylation of IRF3 by noncanonical IKKs, such as TANK-binding kinase 1 (TBK-1) and IKKι (IKKε), leads to its translocation to the nucleus and target gene activation (*188, 189*). TRIF adapter, recruited to TLR3 and TLR4 signalling, associates with TRAF3, which mediates downstream activation of type I interferon production recruiting TBK-1 (*190*).

Plasmacytoid dendritic cells (pDC) are a subset of DCs which are specialized in production of huge amounts of interferon type I in response to viral infection. Signalling through TLR7 and TLR9 leading to activation of type I interferons in those cells is unusual and depends on MyD88 adapter. Phosphorylation of IRF7 in this case, is mediated by IKKα and/or IRAK1, which are recruited to MyD88 via IRAK4, TRAF6 and TRAF3. In addition, also the signalling to NF-κB seems to proceed in a specific way in pDCs. Here, activation of IKK complex downstream of TRAF6 does not involve TAB-TAK1 (*180*).

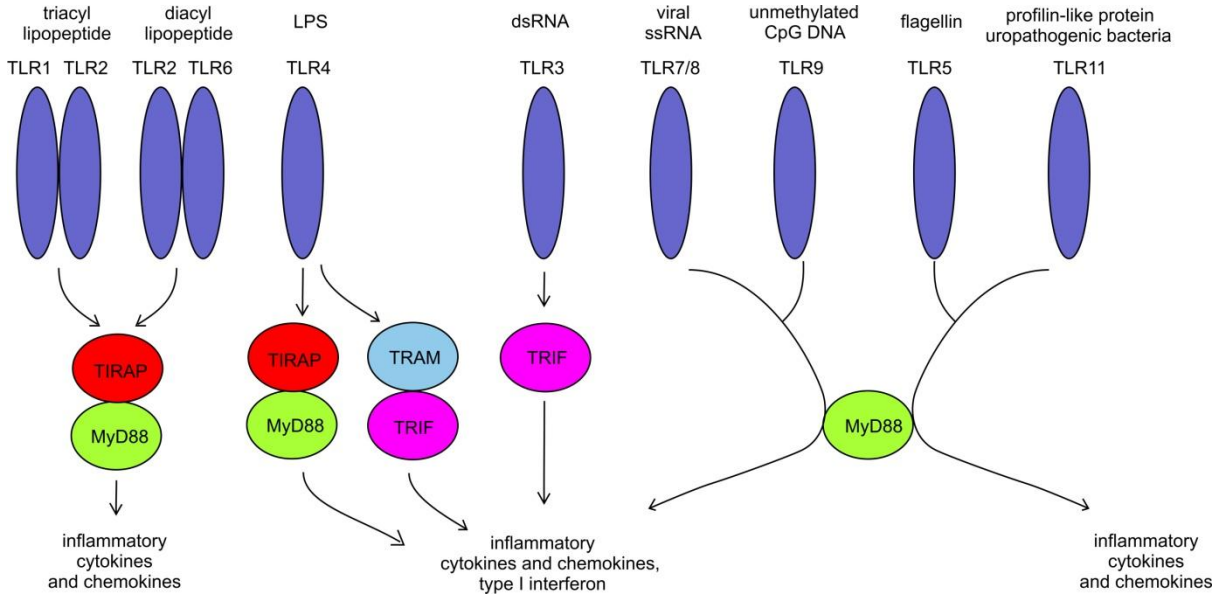

Figure 4. Schematic overview of TLR signalling. Almost all TLRs signal via the MyD88 adapter except TLR3 which stimulation depends exclusively on the TRIF adapter. The endpoint of activation of all TLRs is increased expression of inflammatory cytokines and chemokines, however, TLR3, TLR4, TLR7/8 and TLR9 additionally stimulate type I interferons (modified from Kawai T., Akira S., 2007).

4.3.5. TLR4 pathway

Stimulation of cells with LPS is possible through a series of interactions between different proteins, including LPS binding protein (LBP), CD14, MD-2 and TLR4. CD14 recognizes LPS bound to LBP (free shuttle protein) and facilitates its transfer to the TLR4/MD-2. MD-2 is non-covalently associated with TLR4 and participates in recognition of LPS (*191*). Association of LPS with TLR4/MD-2 results in formation of homodimers of TLR4/MD-2-LPS complexes, followed by recruitment of downstream TIR-domain containing adapters TIRAP/MAL and MyD88 required for the induction of NF-κB and MAPKs. Additionally, upon subsequent TLR4 internalization TRAM and TRIF adapters are recruited, leading to the activation of NF-κB, MAPKs and IRF3 (*180*).

As mentioned above, activation of IRAK kinases takes place upon their recruitment to TLR4 via MyD88 adapter. Ultimately IRAK1 kinase binds to TRAF6 (via TRAF6-binding motifs) thereby stimulating downstream signalling (*192*). TRAF6 is an E3 ubiquitin ligase associated with a dimeric E2 ubiquitin conjugating enzyme complex composed of UBC13 and UEV1A (UBC-like protein) which together drive Lys 63-linked regulatory ubiquitination of target proteins, leading to activation of IKK complex (*193*).

A complex composed of TGFβ-activated kinase (TAK-1) and its binding partners TAK1-binding protein 1 and 2 or 3 (TAB1-3), becomes activated upon recognition of TRAF6-synthetised K63-linked polyubiquitin chains by TAB2/3 through their highly conserved zinc finger (ZnF) domain. This leads to phosphorylation-mediated autocatalytic activation of TAK-1 kinase, which in turn results in activation of IKKβ (*194*).

NEMO/IKKγ and TRAF6 itself belong to known targets of TRAF6-mediated ubiqutination. It has long been thought, that the self-ubiquitination of TRAF6 is crucial for its recognition by TAB2/3 and therefore activation of TAK-1. However, it has recently been shown, that the autoubiquitination of TRAF6 upon treatment with IL-1β is dispensable for the activation of the TAB-TAK1 complex, suggesting a role of other TRAF6 target(s) in the induction of TAK1 and therefore downstream NF-κB and MAPK pathways (*195*). In turn, NEMO/IKKγ recognizes K63-linked ubiquitin chains, as well as becomes K63-ubiquitinated by TRAF6. Intriguingly, ubiquitination of IRAK-1 by TRAF6, but again not TRAF6 autoubiquitination, was suggested to be responsible for interaction of IRAK-1 with NEMO/IKKγ and therefore its TRAF6-mediated activation upon TLR/IL-1R stimulation (*196*). However, unanchored ubiquitin chains (not conjugated to any protein) could also activate TAK1 and IKK complex (*197*).

As mentioned above, NEMO/IKKγ is also a target of covalent modification with K63-linked polyubiquitin chains. Impaired cytokine production following TLR agonist stimulation (including LPS) in macrophages and DCs derived from knock-in mice expressing NEMO/IKKγ which could not be polyubiquitinated at K392 (K399 in human) underscores the importance of this modification in regulating the effect of proinflammatory treatment (*198*). Another study on the role of TRAF6-mediated polyubiquitination of both K399 and K285 of NEMO/IKKγ confirmed, that these ubiquitinations are necessary to accomplish optimal TLR4-mediated activation of NF-κB pathway (*199*).

As additional level of complication in TLR signalling, Tseng and colleagues suggested an important role of TRAF3 in regulation of signalling downstream of both, MyD88 and TRIF adapters. TRAF3 and TRAF6 are recruited to both adapters, however, cellular inhibitors of apoptosis (cIAP1 and cIAP2), two K48-linked ubiquitin

E3 ligases, are recruited only to MyD88. Association between TRAF6 and cIAPs leads to their K63-linked ubiquitination and activation. In turn, active cIAPs target TRAF3 for proteasomal degradation which allows phosphorylation and activation of TAK-1 and downstream MAPKs, but is dispensable for interferon secretion and IKK complex activation. On the other hand, TRAF3 associated with TRIF does not interact with cIAPs and TRAF3-mediated K63-linked autoubiquitination is necessary for induction of type I interferon. Taken together, K48-linked ubiquitination of TRAF3 upon its recruitment to MyD88 is essential for the activation of MAPKs and production of proinflammatory cytokines, but the K63-linked selfubiquitination of TRAF3 activates the interferon type I production in TRIF-mediated signalling (*200*). An overview of TLR4 signalling pathways is shown in Figure 5.

To prevent an excessive proinflammatory response, TLR4 signalling can be negatively regulated on several levels. RP105 (radioprotective 105), ST2L (interleukin 1 receptor, type I, IL-1R1) and SIGIRR (single immunoglobin IL-1R-related molecule) are surface inhibitors of TLR4 signalling. RP-105, a homologue of TLR4, forms complexes with MD-1, a homolog of MD-2. RP-105/MD-1 dimers were shown to interfere with the binding of LPS to TLR4/MD-2. In turn, ST2L (a homologue of IL-1 receptor) may prevent the activation of TLR4 signalling by sequestering MyD88 and TIRAP. Similarly, SIGIRR, another homologue of IL-1R, prevents the interaction between TLR4 and MyD88 adapter through its TIR domain (*191*).

TLR4 signalling can be inhibited also at downstream levels and this includes inhibitory members of the IRAK and TRAF families. For instance, IRAK-M was shown to prevent the dissociation of IRAK1 from MyD88, thereby quenching TLR4 signalling. Similarly, IRAK-2c and MyD88s (splice variants of IRAK-2 and MyD88, respectively, which lack functional domains) have been shown to be inhibitory upon recruitment to TLR4 signalling. Also some members of TRAF family, such as TRAF1 and TRAF4, are known as negative regulators of TLR4 signalling. TRIF-dependent cleavage of TRAF1 mediates the inhibition of TRIF-induced NF-κB and IRF3 activation in a negative feedback loop. TRAF4 on the other hand, was suggested to inhibit NF-κB activation when overexpressed, possibly through its interactions with TRIF and TRAF6 (*191*).

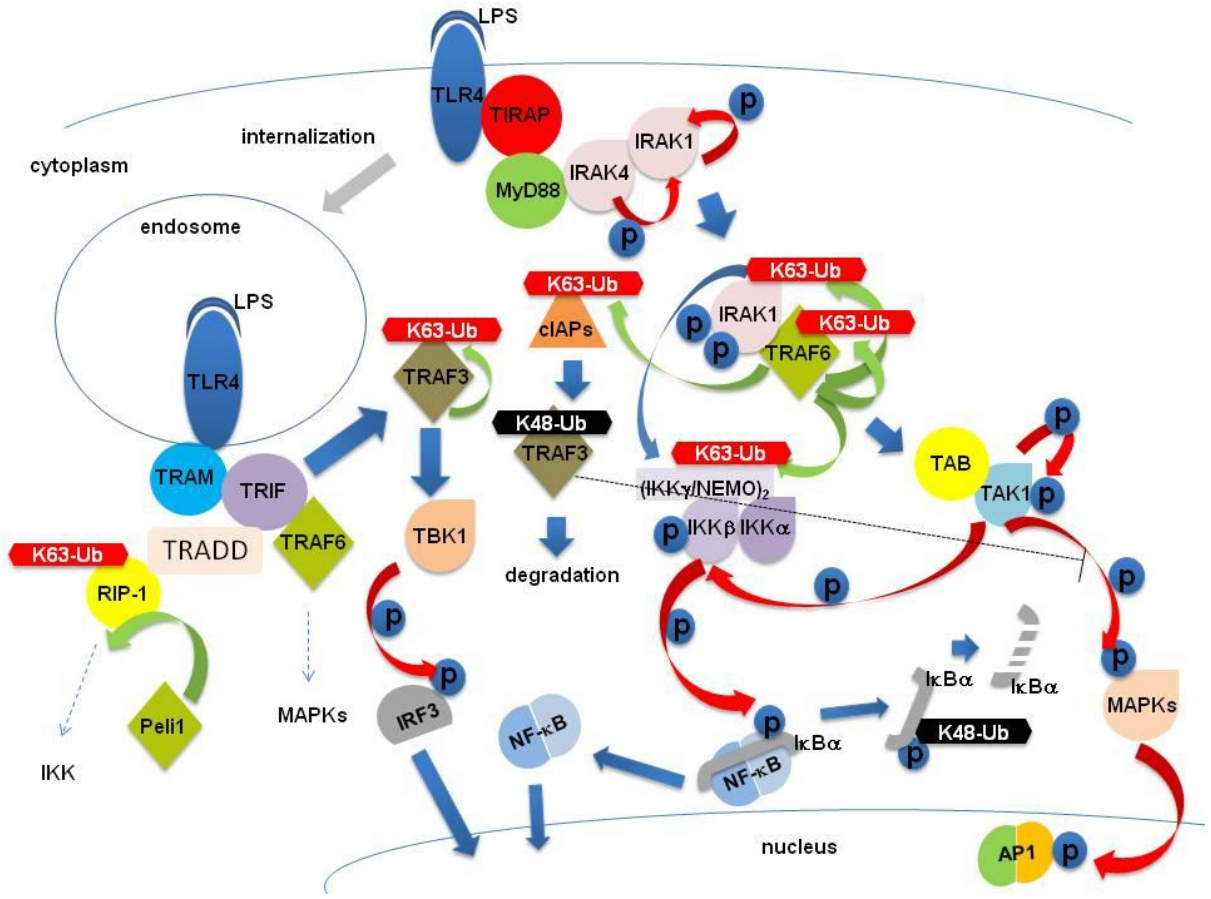

Figure 5. TLR4-mediated signalling pathways. Activation of the MyD88-dependent pathway results in the activation of NF-κB and MAPKs. The subsequent (after TLR4 internalization) recruitment of the TRIF adapter leads to NF-κB, MAPKs and IRF3 activation.

On another level of negative regulation of TLR4 pathway is action of the deubiquitinases (DUBs). A20 (also known as tumor necrosis factor, alpha-induced protein 3 - TNFAIP3) is an example of such a modulator, hydrolyzing K63-linked polyubiquitin chains on NF-κB pathway components. A20 deubiquitination targets are TRAF2, RIP-1 and TRAF6. However, A20 carries also E3 ligase activity, therefore it is classified as an “ubiquitin editing” enzyme. The mode of action of this deubiquitinase depends on its target. Shortly after proinflammatory stimulation, A20 is recruited to RIP-1, mediating the hydrolysis of K63-linked polyubiquitin chains on RIP-1. Subsequently, by K48-linked polyubiquitination, A20 mediates RIP-1 proteasomal degradation. However, although in the case of TRAF6 K63-linked ubiquitination is also inhibited in an A20-dependent manner, this negative regulation seems to depend on the disruption of the E2:E3 ubiquitin enzyme complex, rather than deubiquitination of existing K63-linked polyubiquitin chains. In the next step, the E2 enzymes associated with TRAF6 become modified with K48-linked polyubiquitin

chains and proteasomally degraded. The *Cyld* tumour suppressor gene, which is frequently mutated in familial cylindromatosis (benign tumours of skin appendages), represents an example of another deubiquitinase regulating NF-κB signalling. CYLD removes K63-linked ubiquitin chains of proteins such as TRAF2, RIP-1, TRAF6 and NEMO/IKKγ. Despite sharing some target genes, A20 and CYLD are not redundant. CYLD is responsible for quenching undesired NF-κB activity in unstimulated conditions, whereas A20 inhibits the NF-κB signalling elicited upon proinflammatory stimulation (in a negative feedback loop since A20 is a NF-κB target gene). CYLD-mediated control of NF-κB activation is transiently abrogated by interacting with activated NEMO/IKKγ and phosphorylating of CYLD by IKK, thereby the stimulus-elicited signalling to NF-κB becomes possible (*201*).

4.3.6. The role of NF-κB in the intestinal epithelium

The lumen of the guts is inhabited by trillions of commensal bacteria, whereas the intestinal mucosa contains large number of immune cells. Therefore, the interaction between microbiota and immune system of this site must be precisely controlled to avoid undesired intestinal inflammation (*202*). The epithelium of the gastrointestinal track in an adult person covers approximately 300 m^2 and composes an efficient barrier from the body exterior environment (*203*). Intestinal epithelium response triggered by activation of pattern recognition receptors (PRR), such as toll-like receptors (TLRs) and nod-like receptors (NLRs), by intestinal commensal microbiota include NF-κB-mediated upregulation of regulators of intestinal immune cells activation such as cytokines and chemokines, as well as antimicrobial peptides (*202*). Therefore, except from physically separating mucosal immune system from the luminal content of the guts, intestinal epithelium (mainly the Paneth cells of the small intestine) secreting antimicrobial peptides such as defensins limit the amount of bacterial flora and shape the microbiome composition (*202, 204*). However, NF-κB plays also an important antiapoptotic function in the physiology of gut epithelium. Mice with intestinal epithelium-targeted deletion of NEMO/IKKγ or both kinases IKKα and IKKβ were shown to spontaneously develop sever chronic inflammation in the guts. Deficiency in NF-κB signalling was associated with increased intestinal epithelium apoptosis, decreased synthesis of defensins and excessive flux of commensal bacteria into the mucosa, triggering intestinal inflammation. Furthermore, global knock-out of TNF-α receptor TNFR1 abrogated inflammation induced by the

lack of NEMO/IKKγ expression in intestinal epithelium, an effect at least partially dependent on TNF-α induced epithelial apoptosis and thereby impaired integrity of intestinal epithelium layer in the absence of NEMO/IKKγ (*205*). Although persistent activation of NF-κB signalling in intestinal epithelial cells such as in mice expressing constitutively active IKKβ in this cell type was found to be associated with immune cells infiltration as well as increased production of cytokines and chemokines, animals developed only mild inflammation in the guts (*206*). However, strong sensitization to inflammation elicited by intestinal damage-inducing agent dextran sulfate sodium salt (DSS) could be observed (*206, 207*). Collectively, those studies provided evidences that the proper function of intestinal epithelium is mediated by tight regulation of NF-κB activation level.

4.3.7. Inflammation, NF-κB and cancer

Inflammation may precede the development of malignancy or originate from genetic alterations leading to both inflammation and neoplasia (*24*). The link between chronic inflammatory processes and the development of cancer is well established for example in the case of inflammatory bowel disease (IBD) and colitis-associated cancer (CAC) (*208, 209*). However, activation of inflammatory processes during the course of tumourigenesis can be established due to dysregulated expression of NF-κB pathway members as well as mutations in oncogene/tumour suppressor genes (*210-213*).

Increased activity of NF-κB in tumours is associated with poorer prognosis of cancer patients including colorectal, pancreatic, nasopharyngeal, ovarian, esophageal, lung and cervical malignancies (*214-220*). In addition to protumourigenic effects of inflammatory pathways activation in cancer cells themselves, NF-κB (in concert with signal transducer and activator of transcription 3, STAT3 and HIF-1) drives innate immune cell infiltration into the tumour microenvironment. The infiltrating cells, together with activated cancer and stromal cells, are responsible for further NF-κB, STAT3 and HIF-1α-mediated production of inflammatory mediators such as chemokines, cytokines and prostaglandins. This cumulative action of different cells in cancer microenvironment promotes infiltration of more leucocytes, as well as sustains the activation of leucocytes, stromal and cancer cells, facilitates tumour cell survival and proliferation, and stimulates migration, invasion, angiogenesis,

lymphangiogenesis and metastasis, as well as inhibits adaptive anticancer immunity and contributes to cancer therapy resistance (*24*).

A well known example picturing the *in vivo* roles of cancer cell- and myeloid-derived NF-κB in colorectal carcinogenesis is a study by Greten and colleagues (*221*). In this report, a mouse model with a targeted deletion of IKKβ (unable to activate NF-κB) in colonic epithelial cells reveled decreased colon cancer incidence upon combined treatment with procarcinogen azoxymethane (AOM) and an inducer of colonic inflammation DSS. This effect was associated with increased epithelial apoptosis during early steps of tumourigenesis. Lack of IKKβ, however, did not affect the tumour dimensions (*221*). In line, expression of constitutively active IKKβ (permanently stimulated NF-κB) in enterocytes spontaneously induced tumours in aged mice, as well as strongly promoted AOM/DSS- and *Apc* mutation-induced carcinogenesis (*207*). However, targeted deletion of IKKβ in the myeloid lineage did not affect tumour incidence, but resulted in decreased tumour size, suggesting myeloid cell-derived NF-κB to be an important regulator of cancer growth (*221*).

The key NF-κB-target genes abundantly secreted in cancer microenvironment are TNF-α and IL-1 (inducers of NF-κB), as well as IL-6 (activator of STAT3). The effects of TNF-α, IL-1β and IL-6 in cancer due to activation of mentioned transcription factors are associated with promoting premalignant and malignant cell survival and proliferation, as well as their ability to maintain the cancer-related inflammation (*222*).

Expression of NF-κB-targeted adhesion molecules such as E-selectin, intracellular cell adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) regulates adhesion and migration of the leucocytes, processes involved in physiological inflammation as well as cancer-elicited inflammation (*223*).

NF-κB target genes can be directly involved in modulation of cancer cell survival. Inhibitors of apoptosis, such as BCL-XL, BCL-2, cIAP1, cIAP2, MCL-1, c-FLIP and survivin, are regulated by NF-κB. However, distinct cell types depend on the expression of those regulators to different extent e.g. BCL-XL appears to be a major antiapoptotic factor in enterocytes, whereas c-FLIP seems to be its counterpart in hepatocytes (*224*).

NF-κB participates in the regulation of motility, invasiveness and metastasis. Twist is a known repressor of E-cadherin mediating EMT in cancer metastasis (*225*). Twist was shown to be transcriptionally activated by NF-κB in normal breast epithelia and breast carcinoma cell lines treated with TNF-α. Furthermore, overexpression of p65/RelA was sufficient to induce EMT and the knock-down of Twist attenuated p65/RelA overexpression-mediated EMT, migration and invasiveness, underscoring the role of NF-κB in induction of aggressiveness of breast cancer cells (*226*). Moreover, EMT induction by NF-κB with potential involvement of other transcriptional repressors ZEB-1 and ZEB-2, factors of known role in downregulation of E-cadherin, was shown to occur in non-transformed mammary epithelial cells (*227*).

Furthermore, iNOS/NO, COX-2/prostaglandin E2, as well as MMP2 and MMP9 are other well established NF-κB-regulated players in cancer (*228-231*). Their roles were described in detail in the chapter 4.2.3.

4.3.7.1. Immune cells in carcinogenesis

Solid tumours contain immune cells such as macrophages, neutrophils, mast cells, MDSC, dendritic cells, natural killer (NK) cells, T- and B-cells, coexisting with cancer cells and their stroma (fibroblasts, endothelial cells, pericytes and mesenchymal cells) (*224*).

The mediators of macrophage infiltration from blood monocytes to the tumour are tumour-derived chemokines including chemokine (C-C motif) ligand 2 (CCL2) also known as monocyte chemotactic protein-1 (MCP-1), CCL5 (RENTES), CCL7 (MCP-3), CCL8 (MCP-2), and chemokine (C-X-C motif) ligand 1 (CXCL1), also known as growth-regulated alpha protein (Gro-α). Furthermore, infiltration of macrophages is induced by growth factors such as VEGF, PDGF, TGFβ and macrophage colony-stimulating factor (M-CSF) (*232*). Tumour homed blood monocytes may polarize to dysplay M1-like (classical activation, antitumourigenic) or M2-like (alternative activation, protumourigenic) phenotype, dependent on cytokines present in the tumour microenvironment (*233*). At the site of inflammation, IFN-γ (produced mainly by activated Th1, Tc and NK cells) and LPS elicit activation of M1 phenotype macrophages displaying strong proinflammatory properties. M2 macrophage activation results from stimulation with IL-4 or IL-13 (M2a), immune complexes combined with LPS or IL-1β (M2b) and IL-10, TGF-β or glucocorticoids (M2c) (*234*).

High ratio of M1/M2-like macrophages present in some human cancers including ovarian and gastric tumours, or high content of M1-like macrophages for instance in nonsmall cell lung cancer (NSCLC) and gastric cancer, were reported to positively correlate with a better patient outcome (*25-28*). However, tumour-associated macrophages (TAMs) usually display M2-like protumourigenic, rather than M1-like phenotype of strong antitumor potential. High content of M2-like polarized macrophages in human malignancies such as uveal melanoma and pancreatic cancer have been found to be associated with worse patient prognosis (*29, 30*).

M1 polarized macrophages are well established activators and effectors of the Th1-mediated response, which helps to eliminate intracellular pathogens as well as cancer cells. M2 macrophages are characterized by the ability to inhibit Th1 cells, poor antigen presentation capacity, scavenging of debris, promoting tissue remodeling and angiogenesis, next to their role in promoting Th2 response (and therefore elimination of parasites). M1 macrophages efficiently produce ROS as well as potent proinflammatory cytokines such as IL-1β, TNF-α and IL-6, whereas M2 macrophages produce rather small amounts of ROS and variable levels of inflammatory cytokines. Importantly, M1-activated macrophages display high expression levels of IL-12 and IL-23, and only low production of IL-10. Conversely, the M2-polarized class secretes little IL-12 and IL-23, and higher amounts of IL-10, making polarized macrophages important modulators of the antitumour immune responses (*232*).

IL-12 induces NK cells proliferation and their IFN-γ production, as well as elicits Th cells differentiation towards Th1 (further very efficient producers of IFN-γ), which results in the enhancement of cytotoxic T cells activity. In addition, IFN-γ enhances the tumour antigen presentation in the context of MHC I, contributing to tumoricidal cytotoxic T cells action by potentiating target cell recognition, among other functions. However, IL-23 inhibits infiltration and function of cytotoxic T-cells, although the mechanism of this effect is not fully understood. Therefore, the local balance between the availability of IL-12/IFN-γ and IL-23 in the tumour microenvironment seems to determine the efficiency of immunosurvaillance by cytotoxic T-cells (*235*).

IL-23 secreted by macrophages and cancer cells propagates IL-17-secreting subset of T-cells. Subsequently, secretion of IL-17 and signalling downstream of IL-17 receptor results in expression of proinflammatory effectors including IL-1, IL-6, TNF-α, IL-8 and prostaglandin E2, which further participate in the maintenance of cancer-related inflammation (*235*). Of note, it has recently been shown that IL-17 acting via Act1 ubiquitinase activates TRAF6, which in turn induces NF-κB pathway (*236*).

IL-10, which was discovered first as a Th2-produced factor inhibiting Th1 cytokines secretion, is highly expressed by M2-polarized TAMs, and functions as strong autocrine immunosuppressant attenuating NF-κB activation and IL-12 expression, therefore inhibiting macrophage antitumour activity (*237, 238*).

TAMs further contribute to the inhibition of anticancer immunity by production of CCL17 and CCL22 chemokines, which are responsible for recruitment of suppressor T and Th2 cells (*239*). Furthermore, TGF-β secreted by TAMs plays a role in this aspect of carcinogenesis by inhibiting IFN-γ production in Tc and Th1 cells, as well as by stimulating development of suppressor T cells (*240*).

Further roles of TAMs in cancer embrace promotion of malignant cell survival and proliferation, extracellular matrix (ECM) remodeling, angiogenesis, invasion and metastasis, by secreting huge amounts of effectors such as MMPs, plasmin, uPA, uPAR, TGF-β, VEGF, epidermal growth factor (EGF) and IL-8 (*232*).

Since the functions of macrophages and lymphocytes are inseparably connected to one another, some roles of lymphocytes were described above. However, the importance of distinct T cell subpopulations in cancer underscores the association between their detection in tumour microenvironment and the patient outcome. For instance, in colorectal cancer infiltration of Tc and Th1 cells was associated with better patient prognosis (*241*). On the other hand, the presence of Th17 cells was correlated with poor disease-free survival in another study in the same type of cancer (*242*). Similarly, increased number of suppressor T cells is associated with poor prognosis in several cancers including ovarian, breast, non-small cell lung, hepatocellular, renal, pancreatic, gastric and cervical cancers (*243*). The latter may exert their tumour-promoting effect by inhibiting the activation of Th and Tc cells (*240*).

Tumour-associated neutrophils (TANs) accumulate in the cancer microenvironment owing to cancer-derived expression of chemokines such as IL-8, CXCL1, CXCL2 and CXCL3. By analogy to macrophage polarization phenotypes, there are two distinct types of neutrophils, N1 and N2. TANs of N2 protumourigenic phenotype result from stimulation with TGF-β. Accumulation of TANs in the tumour is associated with increased ROS production and DNA damage, as well as pronounced secretion of VEGF and MMP9 (*232*). Importantly, increase infiltration of neutrophils was found to be associated with adverse patient prognosis for example in colorectal cancer and esophageal squamous cell carcinoma (*244, 245*) .

4.4. Colorectal cancer, a model to study the interplay between hypoxic and inflammatory pathways

Colorectal cancer (CRC) is one of the most common human malignancies, which in 2012 accounted for 1.36 million diagnosed new cases and 694 000 cancer-related deaths worldwide. It is the second most common malignancy in women and the third in men. The risk of developing colorectal cancer by age of 75 amounts to 1.95%, which makes it the highest after malignancies of breast, prostate and lung (*1*).

The luminal side of the small intestine surface forms circular folds (also known as folds of Kerckring) each of which displays further protrusions called villi. Villi are finger-like structures covered mainly with adsorptive enterocytes and mucus-producing goblet cells. Other terminally differentiated cell-types of intestinal epithelium are peptide hormone-secreting endocrine cells and Paneth cells (mainly in small intestine, secreting antimicrobial peptides). Microvilli are cell membrane extensions on epithelial cells which form the “brush border” membrane of the intestine. Intestinal mucosa (or mucous membrane) is formed by a monolayer of epithelial cells placed on lamina propria (underlying loose connective tissue), as well as a layer of smooth muscles called muscularis mucosae. The connective tissue of the mucosa contains capillary vessels, neurons and immune cells, such as mast cells. Submucosal layer, loose connective tissue located below mucosa, contains larger blood vessels and may also carry glands. Submucosa is surrounded by two additional smooth muscle layers, forming muscularis externa (circular and longitudinal muscle layers), and a serous membrane (*79*). Invaginations of the layer of epithelial cells into the underlying lamina propria form intestinal crypts (also known as crypts of Lieberkühn). The crypts contain multipotent stem cells (SCs) located at their bottom, which can give rise to any of the intestinal epithelial cell (*246*). A schematic organization of the intestinal wall is shown in Figure 6. The SCs of colonic crypts divide mostly asymmetrically into one daughter cell being identical to the original cell, and one becoming a progenitor differentiating into other cell types. During divisions progenitors migrate to the top of a crypt reproducing all the terminally differentiated intestinal cell types (*247*). The crypt SCs are the cells from which intestinal cancer originates (*248*).

CRCs are generally divided into sporadic, inherited and colitis-associated cancers. Sporadic cases account for about two thirds of the total number of CRC. In up to one

third of cancer patients moderate inheritance (familial clustering) is observed. Well known hereditary conditions, including familial adenomatous polyposis (FAP) syndrome and Lynch syndrome (hereditary non-polyposis colorectal cancer, HNPCC), are associated with about 3 - 5% of colorectal cancers (*249*). Finally, Colitis-associated cancers (CACs) represent about 1 - 2% of all cases (*250*).

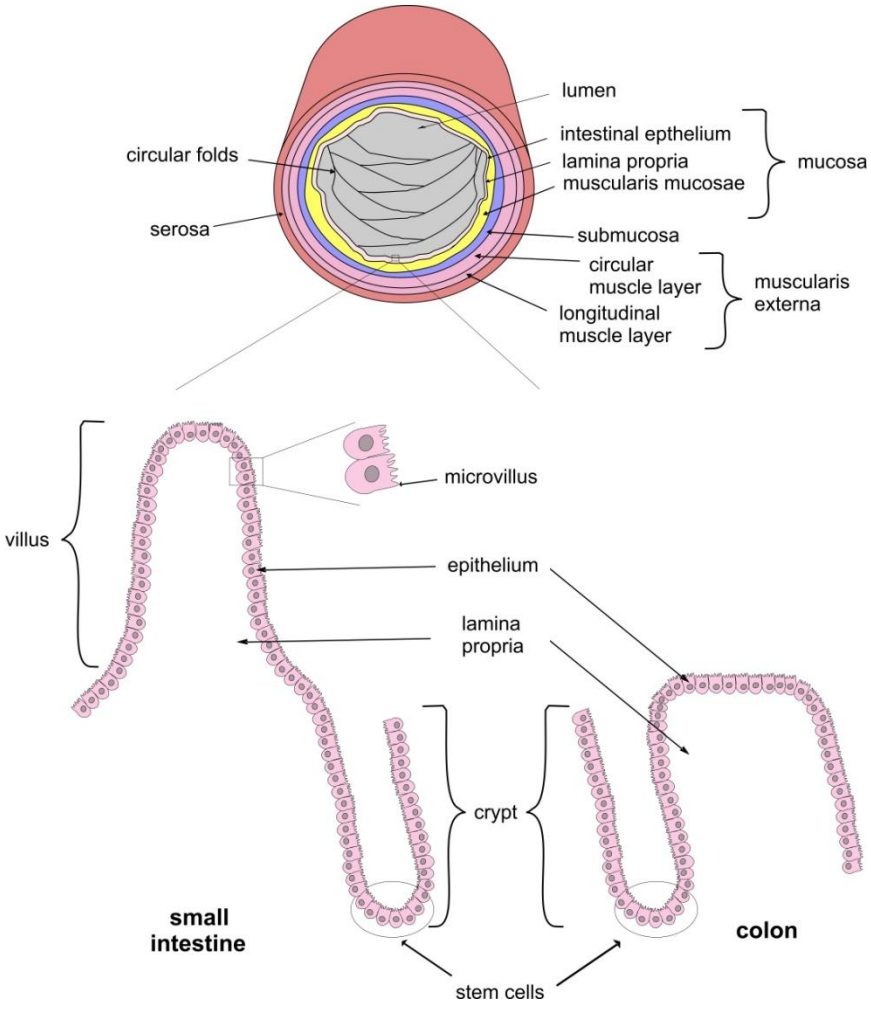

Figure 6. Organization of the intestinal wall. The intestinal wall is organized out of four main layers: mucosa, submucosa, muscularis externa and serosa. Invaginatons of epithelial cell layer towards the underlying lamina propria form intestinal crypts. Lamina propria contains capillary vessels, neurons and immune cells (not shown).

However, although CACs are in minority, the cumulative risk of developing CRC in patients with IBD is up to 18% in ulcerative colitis (UC) and up to 8.3% in Crohn`s disease (CD) 30 years upon IBD diagnosis (*208, 209*). Furthermore, be it the case of

colitis-associated cancer or not, inflammatory infiltrate and expression of proinflammatory cytokines are often present in CRCs (*251*).

The colon of humans is inhabited by about 10^{14} commensal microorganisms, representing a huge range of species diversity (*252*). The importance of microflora in CRC underscores the decreased number and size of chemically-induced tumours in germ-free rats compared to animals harboring regular gut micoroflora (*253*). The main constituents of colon microflora are Gram-negative bacteria, and their outer membrane component LPS is recognized by TLR4 (*254*). Interestingly, TLR4 knock-out mice were protected from tumours in a model of AOM/DSS-induced colon carciongenesis, indicating a role of TLR4 signalling in the development CAC (*255*).

Majority of the TLRs in healthy guts are expressed in antigen presenting cells, whereas intestinal epithelial cells display much lower levels of TLRs (*254*). However, TLR4 receptor as well as its adapter MyD88, were shown to be overexpressed in tumour cells compared to surrounding normal and adenomatous epithelium in human sporadic colon cancers, and expression of TLR4 and MyD88 was associated with poor patient prognosis (*256*). Similarly, over 90% of analysed human CAC expressed high levels of epithelial TLR4 (*257*). Consistently, overexpression of TLR4 in mouse colonic epithelium results in formation of more numerous tumours in response to AOM/DSS-induced carcinogenesis compared to WT mice (*257*).

The presence of hypoxia is characteristic for CRCs and the overexpression of HIF-1α in CRC implies worse patient prognosis (*162, 258*). Similarly, almost all CAC and most of other CRCs display activation of NF-κB and an increase of NF-κB activity in colorectal patients is associated with poorer clinical outcome (*214, 251, 259*). Therefore, colorectal cancer deserves special attention with regard to the possible crosstalk between hypoxic and inflammatory pathways.

4.5. Connections between hypoxia/HIF and inflammation/NF-κB

Some connections between hypoxia/HIF and inflammation/NF-κB were already discussed before. However, the established links between those two responses make more comprehensive picture. It is widely understood that hypoxia may develop at the site of inflammation, for instance owing to increased distance to the vessels (due to edema) or elevated oxygen consumption (linked to ROS production by PMN cells in oxidative burst) (*203*). Inflammatory bowel disease model in mice revealed a prominent increase in the extent of hypoxia compared to healthy animal colonic mucosa, proving inseparable presence of hypoxia during the course of chronic inflammatory condition in the guts (*260*). On the other hand, hypoxia facilitates cell survival and function at the site of inflammation. HIF-1α-mediated induction of glycolysis is indispensable for the maintenance of the cellular ATP levels in myeloid cells, and therefore their inflammatory function (*261*). In addition, survival of neutrophils in hypoxia was shown to be mediated by HIF-1α-dependent NF-κB activation (*262*).

Furthermore, a direct transcriptional regulation of HIF-1α mRNA by NF-κB pathway has been described in different experimental models, including rat macrophage-like (NR8383) cells, human embryonic kidney (HEK) 293, pulmonary artery smooth muscle cells (PASMCs), mouse macrophage-like (RAW247.6) cells, human monocytic cell line (THP-1) (also following differentiation), primary human macrophages, bone marrow-derived macrophages (BMDMs) as well as *in vivo* in the livers and brains of mice (*263-268*). Some groups observed, however, an increase in HIF-1α protein levels following proinflammatory stimulation with no changes of its mRNA abundance, uncovering the existence of other mechanisms of NF-κB-mediated HIF-1α expression regulation (*269-271*). For instance, stabilization of ubiquitinated HIF-1α protein associated with VHL after TNF-α treatment in HEK293 and porcine kidney cells LLC-PK1 (*269*). In other work, stimulation with IL-1β was found to upregulate HIF-1α protein levels in human lung carcinoma A549 cells with the involvement COX-2/prostaglandin E2 synthesis pathway (*270*). Direct transcriptional NF-κB-mediated regulation of HIF-1β mRNA was also reported (*272*). Furthermore, NEMO/IKKγ was shown to interact with HIF-2α (but not HIF-1α), and

therefore enhance its transcriptional activity by recruiting p300/CBP transcriptional co-activators (*273*).

According to some reports hypoxic treatment activates NF-κB pathway. The suggested mechanisms embrace PHD1 inhibition-dependent de-repression of IKKβ kinase in HeLa cells exposed to hypoxia (*274*). Curiously, exposure to hypoxia induced NF-κB in osteosarcoma cell line U2OS, breast carcinoma MDA-MB-231 and nontransformed breast epithelial cell line MCF10A (*275*). This effect was found to be associated with calcium/calmodulin-dependent kinase 2 (CaMK2)-mediated IKK complex activation, which in contrast to other IκBα phosphorylation-inducing pathways, did not lead to the degradation of NF-κB inhibitor, but its sumoylation (*275*). In other study, HPV-encoded E6 protein was reported to mediate inactivation of deubiquitinase CYLD and therefore prolonged hypoxic activation of NF-κB in cell lines infected with high risk HPV (*276*). Moreover, study in neutrophils showed transcriptional targeting of p65/RelA by HIF-1 in hypoxia (*262*).

The effects of proinflammatory stimulation under hypoxia or following PHD enzyme inhibitor treatment differ compared to control conditions. TNF-α-elicited NF-κB activation, in HeLa and HEK293 cells, was shown to be increased in hypoxia compared to normoxia (*274, 277*). However, treatment of Raw264.7 macrophages with the PHD inhibitor dimethyloxalylglycine (DMOG), or shRNA targeting PHD1 or PHD2, suppressed the cytokine expression in response to LPS (*278*). Interestingly, pharmacological hypoxia in rheumatoid arthritis synovial fibroblasts enhanced proinflammatory cytokine production following the treatment with agonists of TLR2, TLR3 and TLR9, but attenuated the cytokine response to TLR4 stimulation (*279*). Finally, Scholz and colleagues showed, that the magnitude of NF-κB activation with IL-1β is decreased after DMOG pretreatment in HeLa cells and *in vivo* in reporter mice, in a manner dependent on combined PHD1 and FIH inhibition (*277*).

There are plenty of data indicating a role of PHDs/HIFs in the modulation of the course of inflammatory bowel disease. Treatment with DMOG resulted in amelioration of DSS-elicited colitis in a manner at least partially dependent on gain of intestinal epithelial antiapoptotic phenotype (*280*). Consistently, $PHD1^{-/-}$ (but not $PHD2^{+/-}$ or $PHD3^{-/-}$) mice were less susceptible to experimentally induced colitis, associated with increased density of colonic epithelial cells due to decreased

apoptosis, and therefore enhanced epithelial barrier function. In line, levels of PHD1 were increased in mice with experimentally induced colitis, whereas in patients suffering from IBD, PHD1 expression positively correlated with the severity of the disease (*281*).

As shown *in vivo* in a mouse model of 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis, extensive mucosal hypoxia and concomitant HIF-1 activation accompanied colitis. Intestinal epithelium-specific depletion of HIF-1 positively correlated with severity of clinical symptoms (such as weight loss, colon shortening, mortality), whereas constitutive activation of HIF-1 was protective in the same model of colitis. The effect of hypoxia/HIF-1 was reflected by the increased expression levels of known epithelial barrier-protective HIF targets, including multidrug resistance 1 (MDR1), intestinal trefoil factor (ITF) and ecto-5'-nucleotidase (CD73) (*260*).

In contrast, the studies by Xue and colleagues showed HIF-2α role in exacerbating experimentally-induced colitis. In this work, HIF-2α levels were shown to be increased in mice with experimentally induced colitis, as well as in patients suffering from ulcerative colitis and Crohn`s disease. Intestinal epithelial depletion of HIF-2α reduced the robustness of inflammation in DSS or *Citrobacter rodentium*-induced mouse models of colitis. Consistently, overexpression of HIF-2α (but not HIF-1α) led to the development of spontaneous colitis, as well as increased susceptibility to DSS or *C. rodentium*-induced colitis. HIF-2α mediated the activation of an inflammatory response through the induction of TNF-α, and TNF-α inhibition abrogated the effect of HIF-2α overexpression on colitis (*282*). Recent report on the association between aircraft travel or journeys to altitudes of more than 2000 meters above sea-level with increased risk of flare-up events in patients with IBD, further suggested that PHD/HIF system might play a context-dependent role in regulation of the inflammation in chronic inflammatory disease in the intestines (*283*).

Interestingly, xenografts of human colon cancer DLD-1 cells with stable HIF-1α knock-down displayed decreased volume and weight, as well as increased infiltration of inflammatory cells (mainly neutrophils), but the tumour angiogenesis was sustained. Mechanistically, in the absence of HIF-1α, hypoxic ROS production was shown to induce NF-κB-driven production of proangiogenic IL-8, which sustained the

angiogenesis (*51*), suggesting the existence of certain level of interconnection of HIF-1 and NF-κB pathways is colon cancer cells. However, the resultant of the concomitant hypoxia/HIF-1α activation and inflammation/NF-κB induction in colon cancer remains currently unknown and needs comprehensive studies, given the fact that both HIF-1α and NF-κB are prominent players in this malignancy.

5. Aims of the thesis

- Study the relevance of hypoxic and inflammatory environmental conditions in colon cancer using mouse colon adenocarcinoma MC-38 cells as a model.

- Analyse the putative response of the HIF pathway to inflammatory stimulation as well as the NF-κB pathway to hypoxia in MC-38 cells.

- Test the requirement of the HIF pathway for NF-κB, and the NF-κB pathway for HIF

- Investigate the potential interplay between hypoxic and inflammatory pathways upon simultaneous stimulation of HIF and NF-κB in MC-38 cells.

6. Results

6.1. Manuscript - Hypoxia attenuates the inflammatory response in colon cancer cells

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ABSTRACT

Two main features common to all solid tumours are tissue hypoxia and inflammation, both of which cause tumour progression, metastasis, therapy resistance and increased mortality. In addition, chronic inflammation is associated with increased cancer risk, as demonstrated for inflammatory bowel disease patients developing colon cancer. Here, we show that MC-38 mouse colon cancer cells contain functional hypoxic (HIF-1α stabilization by low oxygen) and inflammatory (p65/RelA nuclear translocation by LPS) signalling pathways. In contrast to cells of the myeloid lineage, neither was HIF-1 regulated by an inflammatory stimulus nor was NF-κB induced by hypoxia. To analyse the interplay between these two pathways, HIF-1α and p65/RelA were stably knocked-down by viral transduction with shRNA expression vectors, and MC-38 cells were cultured as small 3D tumour spheroids which increased the inflammatory responses without affecting HIF-1α levels. RNA deep sequencing revealed that a surprisingly large fraction of HIF target genes required p65/RelA for hypoxic regulation and a number of p65/RelA target genes required HIF-1α for

inflammatory regulation. Of note, hypoxia attenuated the NF-κB-mediated inflammatory response to LPS. Mechanistically, hypoxia was found to inhibit nuclear translocation of p65/RelA which was associated with enhanced IκB levels, independently of HIF-1α. In summary, these data demonstrate a thight interaction of hypoxic and inflammatory signalling pathways which is clearly different between cancer and myeloid cells.

Key words: hypoxia-inducible factor, inflammatory bowel disease, lipopolysaccharide, NF-κB, tissue oxygenation, tumour hypoxia.

INTRODUCTION

The growth of solid tumours is associated with the presence of tissue hypoxia (insufficient oxygen supply) and accompanied by leukocyte infiltration (inflammation), both of which further affect the metabolism of cancer cells, thereby regulating proliferation, metastasis and therapy efficacy (*284*). Hypoxia-inducible factor (HIF) is the master regulator of the hypoxic response in cancer cells (*81*). HIFs are heterodimeric transcription factors consisting of one out of three different oxygen-sensitive HIFα subunits and a common constitutive HIFβ subunit. A family of prolyl-4-hydroxylase domain (PHD) enzymes covalently modifies two proline residues within the oxygen-dependent degradation (ODD) domain of HIFα subunits. The PHD family comprises three members called PHD1, PHD2 and PHD3. Upon hydroxylation under normoxic conditions, HIFα is bound by the von Hippel-Lindau (VHL) tumour suppressor protein and targeted for proteasomal destruction. The resulting high turnover rate of HIFα subunits allows for a very rapid stabilization under hypoxic conditions, affecting the transcriptional activity of several hundred target genes (*285*). Tumour hypoxia and high HIFα levels are associated with poor prognosis of virtually all cancer types (*286*).

The association between inflammatory bowel disease and colon cancer is one example demonstrating that chronic inflammation pre-disposes to cancer (*287*). Conversely, antiinflammatory drugs decrease the incidence of cancer (*24*). Invading inflammatory cells represent a remarkable proportion of the total tumour mass and they are required for tumour angiogenesis and metastasis (*52*). The nuclear factor (NF)-κB is the central transcription factor activated in inflammation. Canonical NF-κB activation is controlled by inhibitor of NF-κB (IκB) kinases (IKK), mainly IKK-β, needed for phosphorylation-induced degradation of IκB in response to infection and inflammation (*288*). Like HIF-1α, NF-κB can be activated in tumours by extrinsic factors (e.g. autoimmune disease) or intrinsic factors (e.g. oncogenes) (*24*). In cancer cells, NF-κB regulates the expression of genes involved in several processes that play key roles in tumour progression such as proliferation, migration and apoptosis (*289*).

Whether or not NF-κB can also be induced by hypoxia is currently unclear. While some groups reported increased NF-κB activation under hypoxic conditions, others could not detect an increased NF-κB DNA-binding activity in hypoxic nuclear extracts, despite strongly induced HIF levels (*290-294*). This might be explained by the severity and duration of the hypoxic stimulus which may be specific for induction of distinct transcription factors. However, IKK-β has been reported to be a specific PHD1 oxygen sensor target (*291*), and the ankyrin repeats of p105 NF-κB precursor and IκBα have been shown to be hydroxylated by factor inhibiting HIF (FIH), albeit without any functional consequences (*295*). Interestingly, in neutrophils the transcript abundance of p65/RelA was shown to be induced by hypoxia in a HIF-1α-dependent manner. Consistently, hypoxia increased the levels of p65/RelA protein and NF-κB transcriptional activity (*262*).

The gene encoding HIF-1α has been suggested to be a NF-κB target. So far, TNFα, oxidative stress and bacterial infections have been shown to activate *HIF1a* gene transcription via NF-κB (*296-300*). A role for NF-κB in enhancing HIF-dependent tumour progression via inflammatory NF-κB activation has not been investigated yet. To better understand the mutual regulation of hypoxia and inflammation signalling pathways in cancer, we studied their interaction in the mouse colon carcinoma cell line MC-38.

RESULTS

The hypoxic response of MC-38 colon cancer cells is mediated by HIF-1

Mouse MC-38 colon adenocarcinoma cells were exposed to normoxia (21% oxygen) or hypoxia (0.2% oxygen) for 8 to 72 hours using conventional two-dimensional (2D) dish culture, and mRNA levels of HIF-1α and HIF-2α were determined by RT-qPCR. Whereas hypoxia did not upregulate HIF-1α mRNA levels (Figure 1A), it robustly induced HIF-1α protein accumulation (Figure 1B). In contrast, only marginal HIF-2α mRNA levels (Figure 1A) and no HIF-2α protein (data not shown) could be detected, suggesting that HIF-1 rather than HIF-2 mediates the hypoxic response in MC-38 cells. No difference in viability and only slightly decreased proliferation rates were observed under hypoxic conditions (data not shown). Transcript levels of the three

canonical HIF target genes *Glut1*, *Ca9* and *Phd3* were found to be maximally upregulated by 12, 146 and 23-fold, respectively, with different kinetics (Figure 1C).

A proinflammatory stimulus activates the NF-κB pathway in MC-38 colon cancer cells

While the activation of the inflammatory NF-κB pathway by lipopolysaccharide (LPS) is well established mainly in the myeloid lineage, it is less clear whether all components of this pathway are also active in carcinoma cells. Therefore, we stimulated 2D cultured MC-38 colon cancer cells with LPS and analysed p65/RelA subcellular localization by immunofluorescence (Figure 2A) and by immunoblotting of nuclear extracts (Figure 2B). LPS induced nuclear translocation of p65/RelA, demonstrating a functional LPS signalling pathway resulting in NF-κB activation. This conclusion was confirmed by a robust mRNA induction of the canonical NF-κB target genes *Tnfa* and *Cox2* which were significantly induced by 13- and 4-fold, respectively, with different kinetics (Figure 2C). Similarly, IL-6 mRNA was found to be 12-fold upregulated by LPS, though not reaching statistical significance (Figure 2C).

NF-κB is not activated by hypoxia in MC-38 colon cancer cells

It has been suggested that hypoxia directly activates the NF-κB pathway by blocking PHD-mediated hydroxylation of IKK-β, at least in HeLa cervical carcinoma cells (*291*). However, in contrast to LPS treatment, exposure of MC-38 colon carcinoma cells to hypoxia for 0.5 to 4 hours did not induce nuclear translocation of p65/RelA as shown by immunofluorescence (Figure 3A) and immunoblotting of nuclear extracts (Figure 3B), although HIF-1α proteins levels were stabilized under these conditions (see Figure 1B). Consistent with these results, the mRNA levels of the NF-κB target genes *Tnfa*, *Il6* and *Cox2* were not induced in MC-38 cells exposed to hypoxia, but rather showed a small albeit not significant negative trend (Figure 3C). In conclusion, direct hypoxic induction of the NF-κB signalling pathway does not appear to be a general phenomenon in carcinoma cells.

***Hif1a* is not a transcriptional target of NF-κB in MC-38 colon cancer cells**

Several previous reports demonstrated that NF-κB binds to the promoter of the *HIF1A* gene and activates its transcription in a limited number of cell types (*296-299*).

However, no change in HIF-1α mRNA (Figure 4A) or protein (Figure 4B) levels could be detected in MC-38 cells treated with LPS, regardless of the oxygen concentration. These results were confirmed by the absence of any change in the mRNA levels of the canonical HIF target genes *Glut1*, *Ca9* and *Phd3* (compare Figures. 1C and 4C).

Knock-down of p65/RelA does not affect HIF signalling

To investigate whether NF-κB could be involved in baseline rather than in conditional HIF-1α regulation, p65/RelA was knocked-down by RNA interference. MC-38 cells were stably transfected with a shRNA construct targeting p65/RelA, leading to an efficient decrease in p65/RelA mRNA (Figure 5A) and protein (Figure 5B) levels. However, knock-down of p65/RelA neither affected HIF-1α mRNA (Figure 5A) nor protein (Figure 5B) levels. These results were further corroborated by mRNA quantification of the HIF target genes *Glut1*, *Ca9* and *Phd3* which remained unaffected by p65/RelA knock-down under normoxic as well as hypoxic conditions (Figure 5C).

Knock-down of HIF-1α does not affect NF-κB signalling

To investigate the converse possibility, that HIF-1α is involved in baseline rather than conditional NF-κB regulation, HIF-1α was knocked-down by stable shRNA transfection. Because another retroviral vector backbone was used, two different shMOCK controls were generated, termed shMOCK_A (Figure 6) and shMOCK_B (Figure 5) for comparison with shHIF-1α and shp65/RelA, respectively. HIF-1α knock-down efficiency was confirmed on the mRNA (Figure 6A) and protein (Figure 6B) levels. However, HIF-1α knock-down neither affected p65/RelA mRNA levels (Figure 6A) nor total p65/RelA protein levels under normoxic as well as hypoxic conditions (Figure 6B). As shown in Figure 6C, also p65/RelA nuclear translocation following LPS treatment remained unaffected in HIF-1α knock-down MC-38 cells. These results were consistent with the mRNA regulation of the NF-κB target genes *Tnfa* and *Il6* which showed no significant difference following induction by LPS for 1 to 8 hours (Figure 6D).

Unexpected cross-talk between hypoxic and inflammatory pathways

In order to obtain a comprehensive picture of the transcriptome regulation by the interaction between hypoxic and inflammatory pathways, MC-38 cells were further characterized by RNA deep sequencing. Therefore, the cells were cultured as small (approx. 250 μm diameter) three-dimensional (3D) spheroids in hanging drops to more closely recapitulate solid tumour conditions. Of note, 3D spheroid conditions appeared to enhance the inflammatory response compared to conventional 2D sub-confluent conditions in tissue culture plates. Following treatment with LPS, the TNF-α mRNA induction rose from 7-fold in 2D to 21-fold in 3D (supplementary Figure S1A). Although we and others previously found hypoxic conditions in the core of larger tumour spheroids (*301*), HIF-1α remained largely unaffected in the relatively small MC-38 3D spheroid cultures (supplementary Figure S1B).

Stable shHIF-1α, shp65/RelA or shMOCK MC-38 cells were cultured in 3D for 48 hours, exposed to 0.2% oxygen for 8 hours and treated with 1 μg/ml LPS for the last 1 hour of the hypoxic exposure. RNA deep sequencing and subsequent analysis was performed as described in the Materials and Methods section. Thresholds for induction and repression were chosen to be at least 1.5-fold or 0.67-fold, respectively. In the case of cells transfected with shHIF-1α or shp65/RelA, "repression" and "induction" in comparison with shMOCK-transfected cells refers to genes positively and negatively, respectively, regulated by HIF-1α and/or p65/RelA (supplementary Table S1).

In summary, for the correct regulation of the majority of the hypoxically induced genes not only a functional HIF pathway (72%) but, unexpectedly, also a functional NF-κB pathway (77%) was required. While regulation of 59% of the genes induced by the inflammatory stimulus was dependent on NF-κB signalling, only 18% required the HIF pathway. Finally, the number of genes induced by LPS under normoxic conditions was reduced by almost 50% under hypoxic conditions, suggesting that hypoxia attenuates the inflammatory response of MC-38 cells.

Suppression of proinflammatory gene expression by HIF-1

RNA sequencing revealed several mRNAs coding for proinflammatory proteins, including CCL20, CXCL5, CSF2 and TNF-α, whose expression was repressed by HIF-1. Therefore, we validated the regulation of these genes by RT-qPCR analysis of independent experiments (Figure 7). Consistent with the RNA sequencing results, hypoxia attenuated the LPS-dependent stimulation of all above mentioned mRNAs in a HIF-1α dependent manner.

Hypoxia impairs LPS-stimulated NF-κB signalling by affecting the levels of IκBα

Since nuclear translocation is the key event in NF-κB activation, p65/RelA subcellular localization was analysed by immunofluorescence. Interestingly, while LPS strongly induced p65/RelA nuclear translocation (Figure 8A, left), this was blocked by simultaneous hypoxic exposure in HIF-1α wild-type as well as knock-down cells (Figure 8A, right). A similar result was obtained with 3D MC-38 cultures (supplementary Figure S2). To elucidate the mechanism of impaired p65/RelA nuclear translocation, p65/RelA translocation and IκBα levels were analysed in the same experiment (Figure 8B). A quantitative comparison of the number of cells with nuclear p65/RelA localization following LPS stimulation revealed an inverse association with IκBα. Consistent with the attenuated NF-κB response, more cells were IκBα positive under hypoxic conditions (Figure 8C).

DISCUSSION

Mortality of colorectal cancer patients has been demonstrated previously to be associated with high HIF-1α but not HIF-2α levels (*162, 302*). The mouse model of colorectal cancer used in this study shows relevant features: HIF-2α mRNA levels in MC-38 colon adenocarcinoma cells were at least two orders of magnitude lower than the HIF-1α mRNA levels. Moreover, no HIF-2α protein could be detected under hypoxic conditions (data not shown) and the hypoxic response of the canonical HIF target genes *Ca9* and *Phd3* was almost fully abrogated in shHIF-1α cells, demonstrating the lack of redundancy of the HIF response in these cells (supplementary Figure S3). This finding is in line with a recent report demonstrating that the *HIF2A*/*EPAS1* but not the *HIF1A* gene is epigenetically silenced in colonic

adenocarcinoma specimens when compared with non-neoplastic tissue (*303*). Therefore, shHIF-1α knock-down was sufficient to allow for the investigation of the role of the HIF signalling pathway in MC-38 cells.

NF-κB plays an important role during colorectal cancer progression and NF-κB has been shown to be a prognostic factor negatively associated with survival in patients with metastatic colorectal cancer (*214, 302*) Colorectal cancer cells are likely to be exposed to bacterial LPS, derived from the gut microbiota, which is known to activate NF-κB via Toll-like receptor (TLR) 4. Both TLR4 and its adapter MyD88 are increased in human sporadic colon cancer compared to surrounding normal and adenomatous epithelium, and they are associated with poor patient prognosis (*256*), suggesting that the LPS-TLR4-NF-κB pathway plays a functional role in colon cancer. The epithelial MC-38 colon cancer cells used in this study robustly activated NF-κB signalling in response to treatment with LPS, making them a suitable model to study the cross-talk between the hypoxic and the inflammatory pathways in colon cancer.

Although MC-38 cells responded to LPS with nuclear p65/RelA translocation as well as NF-κB target gene expression, no transcriptional induction of HIF-1α mRNA could be observed, although it was previously reported to occur in several other cellular systems (*296-300*). Even under 3D culture conditions, where we generally observed a stronger inflammatory response than in 2D cultures, no upregulation of HIF-1α mRNA upon NF-κB activation could be detected (data not shown). Similarly, four additional human colon cancer cell lines (COLO-741, HT-29, SW-620 and CX-1) showed no HIF-1α mRNA upregulation following LPS treatment (data not shown). In contrast, we observed a 6-fold upregulation of HIF-1α mRNA in primary mouse peritoneal macrophages following LPS stimulation (supplementary Figure S4), strongly suggesting that transcriptional HIF-1α regulation by NF-κB is a cell type-specific phenomenon. However, the additionally required factors that confer HIF-1α induction by NF-κB are currently unknown.

In contrast to several previous reports suggesting hypoxic NF-κB induction, (*290, 291, 293, 294*) we found no increase in NF-κB activity upon acute (up to 4 hours) or chronic (up to 72 hours) hypoxic exposure of MC-38 cells. Of note, only four of 32

different cancer cell lines have been shown to sustainably upregulate NF-κB activity during a prolonged (48 hours) exposure to hypoxia, and this effect required the HPV-encoded E6 protein. Just a few HPV-negative cell lines showed a moderate and transient (up to 3 hours) induction of NF-κB activity upon exposure to hypoxia (*292*). PHD oxygen sensing enzymes have been shown to be both positively and negatively implicated in NF-κB activation. PHD1 negatively regulates IκB kinase-β in HeLa cells, and PHD3 is a negative regulator of NF-κB in skeletal myoblast differentiation (*291, 304*). On the other hand, LPS-induced cytokine expression in macrophages was suppressed by treatment with the PHD inhibitor dimethyloxalylglycine or siRNA-mediated knockdown of PHD1 and PHD2 (*305*).

Rather than inducing NF-κB, however, hypoxia attenuated the proinflammatory response to LPS in MC-38 cells cultured under 2D and 3D conditions. RNA deep sequencing revealed a role for HIF-1α in the decreased hypoxic induction of some but not of all NF-κB target genes. A direct repressive HIF function is unlikely because genome-wide DNA association studies combined with transcriptional profiling and assessment of histone marks clearly revealed a transcriptional enhancer but no repressor function of both HIF-1α and HIF-2α (*306*). Quite unexpectedly, hypoxia inhibited the LPS-mediated decrease in IκBα levels in a HIF-independent manner, resulting in decreased p65/RelA nuclear translocation. While these findings, at least partially explain the attenuated inflammatory response, we currently do have no explanation how hypoxia influences IκBα. It has recently been suggested that inhibition of the oxygen-dependent PHD1 or FIH protein hydroxylases blocks distal IL-1β signalling components of the TRAF6 complex involved in NF-κB activation (*307*). Whether similar mechanisms are involved in the hypoxic attenuation of the LPS response needs to be investigated.

Both, the hypoxic and the inflammatory pathways are increasingly being recognized for cancer diagnosis as well as for tumour therapy (*284*). Our results demonstrate a complex interplay between these two pathways in colon cancer cells. Whereas direct transcriptional cross-talks between HIF-1α and p65/RelA could not be confirmed in this cell type, strong indirect interactions between the two pathways could be

demonstrated, suggesting that these two microenvironmental stimuli should always be considered simultaneously when designing novel approaches to tumour therapy.

MATERIALS AND METHODS

Cell culture

Mouse colon adenocarcinoma MC-38 GFP (if not indicated otherwise, hereafter referred to as "MC-38") cells (*308*) were cultured in high glucose DMEM medium (Sigma, St. Louis, MO, USA; or Gibco, Carlsbad, CA, USA) supplemented with 10% FCS (Gibco), 50 IU/ml penicillin and 100 μg/ml streptomycin. MC-38 3D spheroid cultures were grown in hanging drops in 60-well microtest plates (Greiner, Frickenhausen, Germany) by seeding 2500 cells/25 μl drop 48 hours before the experiment. For hypoxia experiments, cells were grown in a gas-controlled workstation (Invivo$_2$ 400, Ruskinn Technologies, Pencoed, UK). For inflammatory stimulation, MC-38 cells were treated with 1 μg/ml LPS derived from *E.coli* (Sigma).

mRNA quantification

Total cellular RNA was extracted as described(*309*) or by using the Nucleospin RNA II extraction kit (Macherey-Nagel, Düren, Germany). cDNA was synthesized from 1-2 μg of total RNA using Affinity Script reverse transcriptase (RT) (Agilent Technologies, Santa Clara, CA, USA). Quantitative (q) PCR was performed using SybrGreen master mix (Sigma) on a MX3000P light cycler (Agilent Technologies). Primers were purchased from Mycrosynth (Balgach, Switzerland) and are listed in supplementary Table S2. RNA sample quality and equal inputs were assessed by RT-qPCR quantification of ribosomal protein S12 mRNA, and all data were expressed as ratios over S12 mRNA.

Protein extraction and immunoblotting

Cells were washed twice with ice-cold PBS by centrifugation for 3 minutes at 1200 rpm. For total protein extraction, cells were resuspended in lysis buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 400 mM NaCl, 0.1% NP-40, freshly added 1 mM PMSF and Sigma's Protease Inhibitor Cocktail). For nuclear protein extraction, cells were resuspended in hypotonic buffer (10 mM HEPES/KOH pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA with freshly added 1 mM PMSF). After 15 minutes on ice, NP-40 was added to a final concentration of 0.1% and the samples were immediately

vortexed for 20 seconds. Nuclei were pelleted for 30 seconds at 5000 rpm, washed with hypotonic buffer and extracted on ice in hypertonic extraction buffer (20 mM HEPES/KOH pH 7.9, 400 mM NaCl, 1mM EDTA, 1mM EGTA and protease inhibitors) for 10 minutes. Proteins were separated by 10% SDS-PAGE, electrotransferred to PVDF membranes and detected using anti-p65/RelA (Santa Cruz Biotechnology, Dallas, TX, USA), anti-HIF-1α (Novus Biologicals, Littletone, CO, USA), anti-HDAC1 (Abcam, Cambridge, UK), anti-SP1 (Santa Cruz Biotechnology) or anti-β-actin (Sigma) antibodies.

Immunofluorescence microscopy

Following fixation with 4% paraformaldehyde for 30 minutes at room temperature, MC-38 cells were washed twice with PBS, permeabilized with 0.1% saponin, free aldehyde groups quenched with 20 mM glycine, and unspecific binding sites blocked with 10% FCS for 30 minutes. Proteins were detected by incubation for 1 hour at 37°C with anti-p65/RelA (Santa Cruz Biotechnology) or anti-IκBα (Cell Signalling Technology, Denvers, MA, USA) antibodies. After washing with PBS, the respective secondary antibodies labelled with Cy3 (Jackson Immunoresearch, West Grove, PA, USA), Alexa488 or Alexa568 (Molecular Probes, Carlsbad, CA, USA) were applied, and nuclei were counter-stained with DAPI. Samples were mounted using Mowiol (Millipore, Darmstadt, Germany) and analysed by fluorescence microscopy. Spheroids were fixed in 10% formalin for 30 minutes at room temperature and processed for histological analysis using previously described techniques.(*310*) Dewaxed paraffin sections (5 μm) were rehydrated, blocked with 10% normal goat serum and processed as described above.

RNA interference

Knock-down of specific mRNAs was achieved by lentiviral transduction of short hairpin RNA (shRNA) vectors driven by the U6 promoter in the pLKO.1-puro plasmid (Sigma). Vectors targeting mouse HIF-1α (shHIF1α) and non-target controls (shMOCK_A) were purchased from Sigma. To construct the shp65 vector, the oligonucleotides 5'-gatccccagggcaaactgtagagtcattcaagagatgactctacagtttgccctttttggaaa-3' and 5'-agcttttccaaaaaagggcaaactgtagagtcatctcttgaatgactctacagtttgccctggg-3' were annealed, phosphorylated and ligated into the pSUPER vector. A BamHI/SalI fragment of the

H1-RNA promoter was ligated into the pRDI292 vector (kind gift from P. O. Hassa, Zurich, Switzerland) and used for expression of shp65.

RNA sequencing

RNA was extracted and pooled from 3 independent 3D-cultures grown under hypoxic or normoxic conditions. Ribosomal RNA in the pooled RNA was depleted using Encore Complete RNA-Seq Library Systems (NuGEN, San Carlos, CA, USA) and RNA was sequenced using an Illumina HiSeq 2000 sequencer (San Diego, CA, USA) at the Functional Genomics Center Zurich. Isoform and gene expression levels were computed with RSEM (Version 1.2.0; http://www.biomedcentral.com/1471-2105/12/323). RSEM was run in stranded mode with the additional option to estimate the distribution of the read start positions. The expression levels (RSEM's posterior estimate of the read counts per transcript normalized for gene length) were normalized across samples using the geometric mean of all expression signals. To attenuate expression ratios for low abundance genes, a fixed value of 5 was added to all expression values before computing the log-ratio. Threshold levels for hypoxia- or LPS-dependent up- or downregulation were set at >1.5 or <0.67, respectively, as were the threshold levels for HIF-1α or p65/RelA dependency of up- or down-regulated gene expression when compared to the respective shMOCK controls. Only genes with a minimal expression level of 10 were considered biologically relevant.

Statistical analysis

Unless indicated otherwise, all experiments were repeated at least three times independently. Data are shown as mean values ± SD. Student's t-tests were applied to analyse the data with a *p*-value < 0.05 considered to be statistically significant.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

This work was supported by the Swiss National Science Foundation (grants PDFMP3_127315 and 31003A_146203) and the KFSP *Tumour Oxygenation* of the University of Zurich.

SUPPLEMENTARY INFORMATION

Supplementary Information accompanies the paper on the *Oncogene* website (http://www.nature.com/onc).

FIGURE LEGENDS

Figure 1. Hypoxic response of MC-38 cells. (**A**) Time-dependent RT-qPCR quantification of HIF-1α and HIF-2α mRNA levels following exposure to hypoxia as indicated. (**B**) Kinetics of hypoxic HIF-1α protein accumulation analysed by immunoblotting of 50 μg total protein extracts. (**C**) Kinetics of hypoxic induction of canonical HIF target genes quantified by RT-qPCR of GLUT-1, CAIX and PHD3 mRNA. Shown are mean values ± SD of mRNA ratios relative to the constitutively expressed ribosomal protein S12 mRNA levels of n = 3 independent experiments; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (Student's t-test).

Figure 2. Inflammatory response of MC-38 cells. (**A**) Immunofluorescence microscopy of p65/RelA in MC-38 cells following treatment with 1 μg/ml LPS for 40 minutes. (**B**) Immunoblot detection of p65/RelA and HDAC1 in nuclear extracts derived from MC-38 cells following stimulation with 1 μg/ml LPS for 1 hour (n = 2 independent experiments). (**C**) Kinetics of inflammatory induction (1 μg/ml LPS for the time periods indicated) of canonical NF-κB target genes quantified by RT-qPCR of TNF-α, IL-6 and COX-2 mRNA. Shown are mean values ± SD of mRNA ratios relative to the constitutively expressed ribosomal protein S12 mRNA levels of n = 3 to 4 independent experiments; **, $p < 0.01$ (Student's t-test).

Figure 3. NF-κB signalling under hypoxic conditions. (**A**) Immunofluorescence microscopy of p65/RelA in MC-38 cells following exposure to 0.2% oxygen for 0.5 to 4 hours. Treatment with 1 μg/ml LPS for 1 hour served as positive control. (**B**) Immunoblot detection p65/RelA and the constitutive Sp1 transcription factors in MC-38 nuclear extracts following exposure to 1 μg/ml LPS or 0.2% oxygen for 1 to 12 hours. (**C**) RT-qPCR analysis of the regulation of the canonical NF-κB target genes *Tnfa*, *Il6* and *Cox2* following hypoxic exposure.

Figure 4. HIF signalling under inflammatory conditions. (**A**) RT-qPCR analysis of the HIF-1α mRNA response to LPS treatment in MC-38 cells. (**B**) Kinetics of the HIF-1α protein response to LPS and/or hypoxia as assessed by immunoblotting of 50 μg total cell extracts. β-Actin served as loading control. (**C**) RT-qPCR analysis of the effects of LPS treatment on the canonical HIF targets gene *Glut1*, *Ca9* and *Phd3*.

Figure 5. HIF signalling in p65/RelA knock-down cells. (**A**) RT-qPCR analysis of HIF-1α and p65/RelA mRNA in MC-38 cells stably transfected with shp65/RelA or shMOCK_B negative control constructs. (**B**) Immunoblotting of HIF-1α and p65/RelA protein after 8 to 72 hours of hypoxic exposure of shMOCK_B or shp65/RelA MC-38 cells. β-Actin served as loading control. (**C**) RT-qPCR analysis of the canonical HIF target genes *Glut1*, *Ca9* and *Phd3* in shMOCK_B or shp65/RelA MC-38 cells cultured under normoxic or hypoxic conditions as indicated.

Figure 6. p65/RelA signalling in HIF-1α knock-down cells. (**A**) RT-qPCR analysis of HIF-1α and p65/RelA mRNA in MC-38 cells stably transfected with shHIF1α or shMOCK_A control constructs. (**B**) Immunoblotting of HIF-1α and p65/RelA protein after 8 hours of hypoxic exposure of shMOCK_A or shHIF1α MC-38 cells. β-Actin served as loading control. (**C**) Immunofluorescence microscopy of p65/RelA in shMOCK_A or shHIF-1α MC-38 cells treated with 1 μg/ml LPS for 40 minutes. (**D**) RT-qPCR analysis of the canonical NF-κB target genes *Tnfa* and *Il6* in shMOCK_A or shHIF-1α MC-38 cells upon treatment with LPS for 1 to 8 hours.

Figure 7. Validation of the RNA sequencing data. MC-38 cells were grown as spheroids in hanging drop cultures for 48 hours before exposure to hypoxia (0.2% oxygen) for 8 hours and treatment with 1 μg/ml LPS for the last 1 hour of normoxic or hypoxic cell culture. CCL20, CXCL5, CSF2 and TNF-α mRNA levels were quantified by RT-qPCR. Shown are mean values normalized to the mean of each experiment ± SD of mRNA ratios relative to the constitutively expressed ribosomal protein S12 mRNA levels of n = 3 to 4 independent experiments. Student's t-test were performed to evaluate the effects of hypoxia on LPS stimulation: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Figure 8. Inverse regulation of NF-κB and IκB under hypoxic conditions. (**A**) Immunofluorescence microscopy of p65/RelA in shMOCK_A and shHIF-1α MC-38 cells following exposure to 0.2% oxygen for 8 hours and/or 1 μg/ml LPS for the last 40 minutes before harvesting. (**B**) Immunofluorescence microscopy of IκBα and p65/RelA in MC-38 cells (without GFP) exposed to 0.2% oxygen for 8 hours and/or 1 μg/ml LPS for the last 30 minutes before harvesting. (**C**) For fractional quantification of the results shown in (**B**), at least 200 cells of each experiment were classified according to the subcellular localization of p65/RelA and the expression of IκB was quantified with use of ImageJ.

SUPPLEMENTARY INFORMATION

Table S1. Gene regulation revealed by RNA sequencing.

Table S2. Primers used for real-time PCR.

Figure S1. Spheroid cultures of MC-38 colon carcinoma cells. MC-38 cell were either cultured as sub-confluent two-dimensional monolayers in tissue culture plates ("2D") or as three-dimensional spheroids in hanging drops ("3D") for 48 hours before the experiment was started. (**A**) Both 2D and 3D cultures were stimulated with 1 μg/ml LPS for 1 hour and the TNF-α mRNA levels were determined by RT-qPCR. (**B**) Cells were exposed to hypoxia (3% or 0.2% O_2) for 8 hours and/or 1 μg/ml LPS for the last 2 hours before harvesting. HIF-1α protein was detected by immunoblotting of 50 μg of total protein extracts. β-Actin served as loading control.

Figure S2. Nuclear translocation of p65/RelA in hypoxic spheroid cultures. Immunofluorescence microscopy of p65/RelA in MC-38 cells grown as spheroids in hanging drop cultures for 48 hours before exposure to hypoxia (0.2% oxygen) for 8 hours and/or 1 μg/ml LPS for the last 40 minutes before harvesting.

Figure S3. CAIX and PHD3 mRNA levels in shHIF-1α MC-38 cells. Hypoxic induction of the canonical HIF target genes *Ca9* and *Phd3* was quantified by RT-qPCR. Shown are mean values ± SD of mRNA ratios relative to the constitutively expressed ribosomal protein S12 mRNA levels of n = 3 to 4 independent experiments.

Figure S4. Inflammatory response of macrophages. Transcript levels of TNF-α and HIF-1α were determined by RT-qPCR in mouse primary peritoneal macrophages following stimulation with 100 ng/ml LPS for 8 hours.

FIGURES AND TABLES

Fig.1

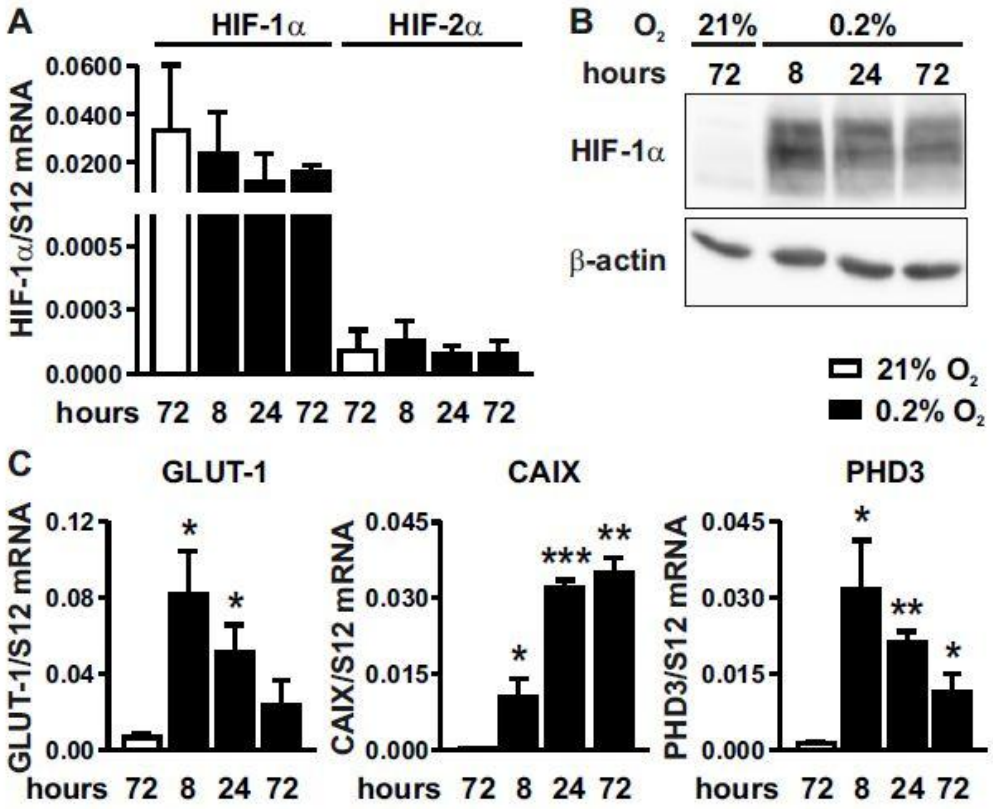


Fig.2

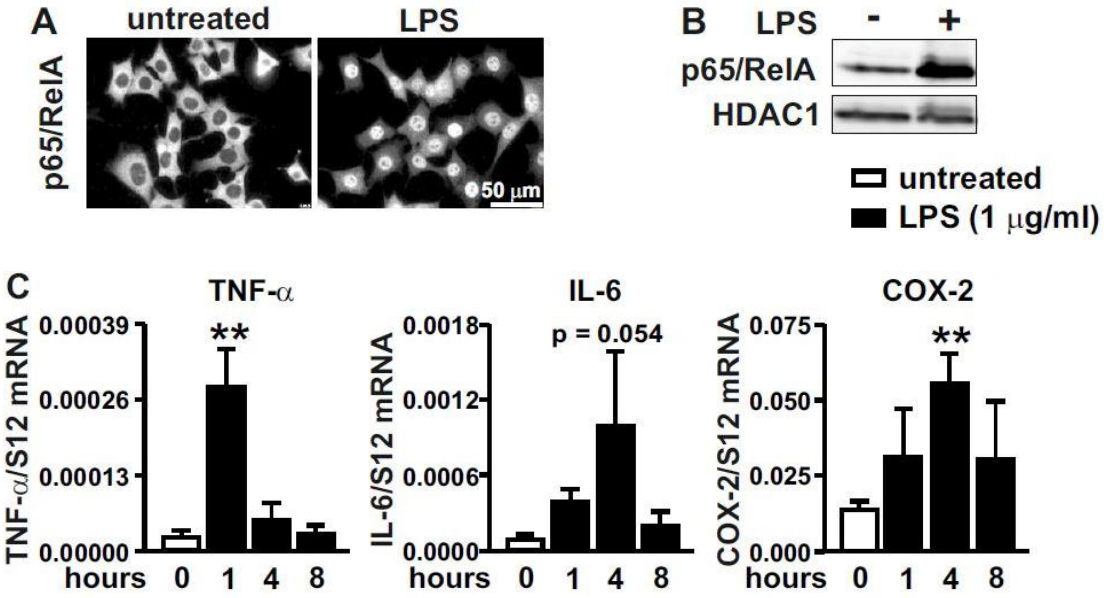


Fig.3

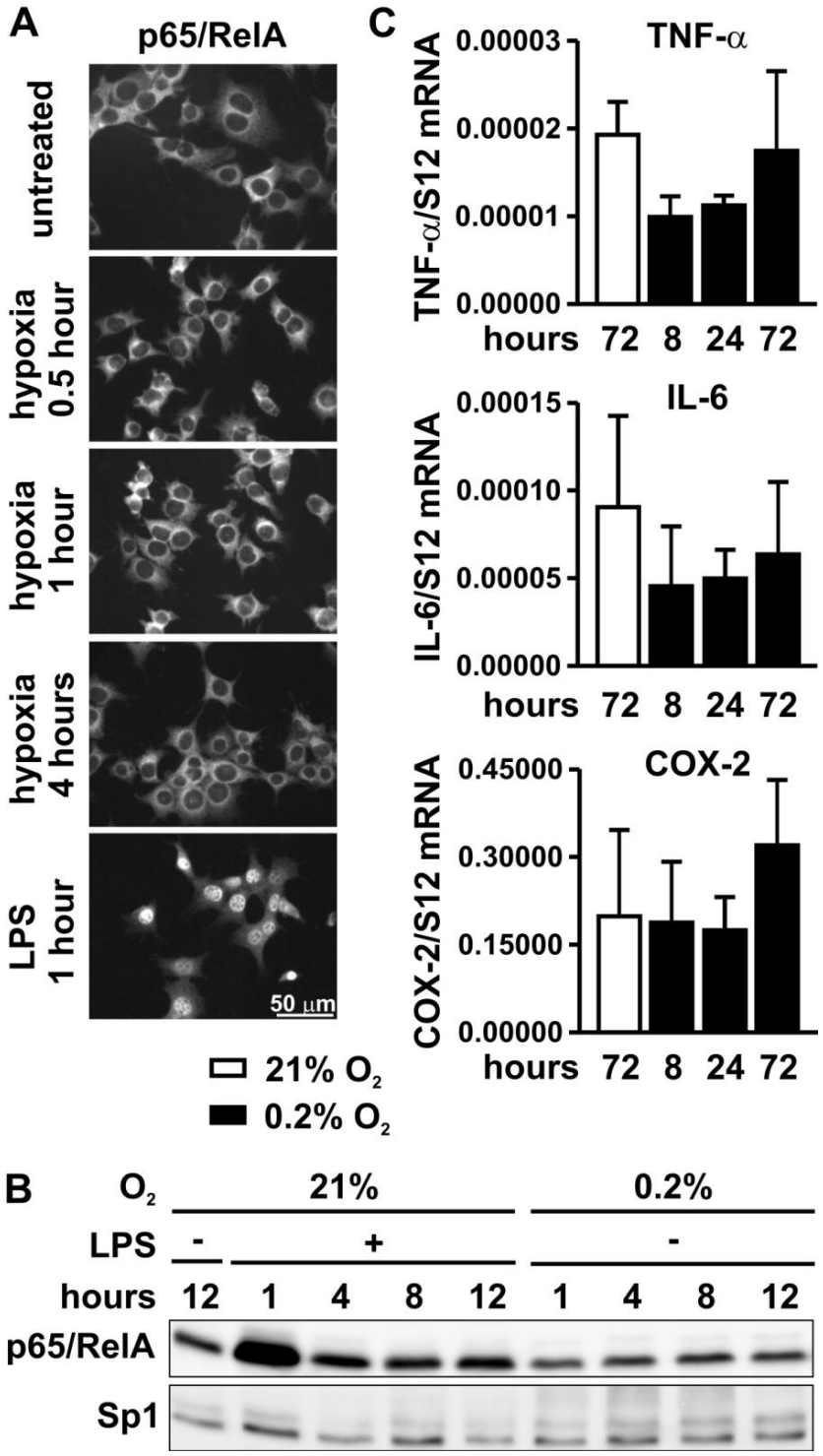

A
p65/RelA
untreated
hypoxia 0.5 hour
hypoxia 1 hour
hypoxia 4 hours
LPS 1 hour
50 μm
C
TNF-α
TNF-α/S12 mRNA
0.00003
0.00002
0.00001
0.00000
hours 72 8 24 72
IL-6
IL-6/S12 mRNA
0.00015
0.00010
0.00005
0.00000
hours 72 8 24 72
COX-2
COX-2/S12 mRNA
0.45000
0.30000
0.15000
0.00000
hours 72 8 24 72
21% O2
0.2% O2
B
O2 21% 0.2%
LPS - + -
hours 12 1 4 8 12 1 4 8 12
p65/RelA
Sp1

Fig.4

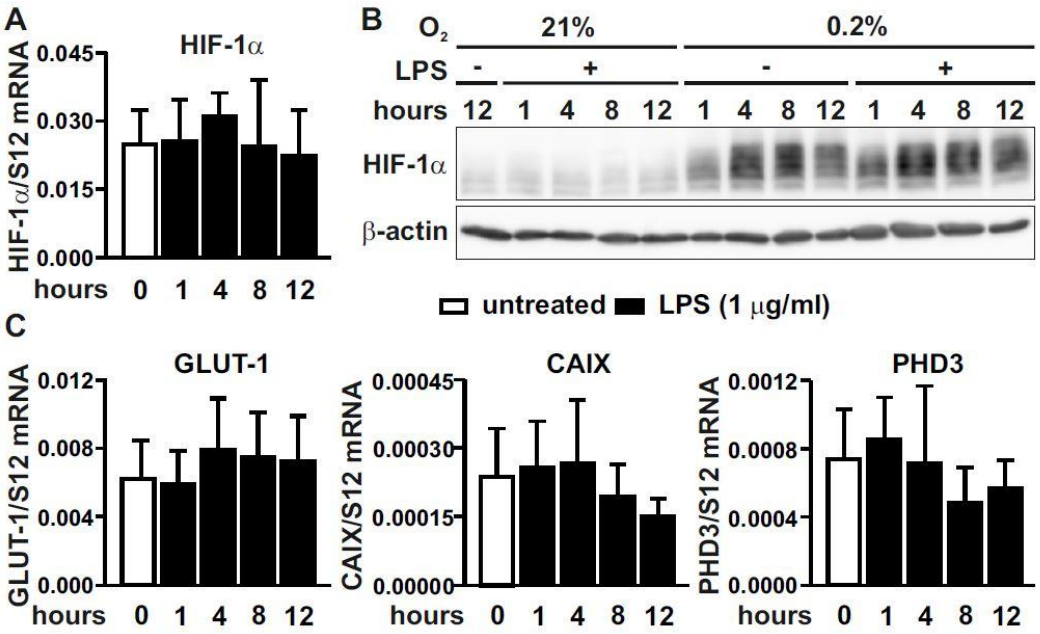

A
HIF-1α
HIF-1α/S12 mRNA
0.045
0.030
0.015
0.000
hours 0 1 4 8 12
B
O2 21% 0.2%
LPS - + - +
hours 12 1 4 8 12 1 4 8 12 1 4 8 12
HIF-1α
β-actin
untreated LPS (1 μg/ml)
C
GLUT-1
GLUT-1/S12 mRNA
0.012
0.008
0.004
0.000
hours 0 1 4 8 12
CAIX
CAIX/S12 mRNA
0.00045
0.00030
0.00015
0.00000
hours 0 1 4 8 12
PHD3
PHD3/S12 mRNA
0.0012
0.0008
0.0004
0.0000
hours 0 1 4 8 12

Fig.5

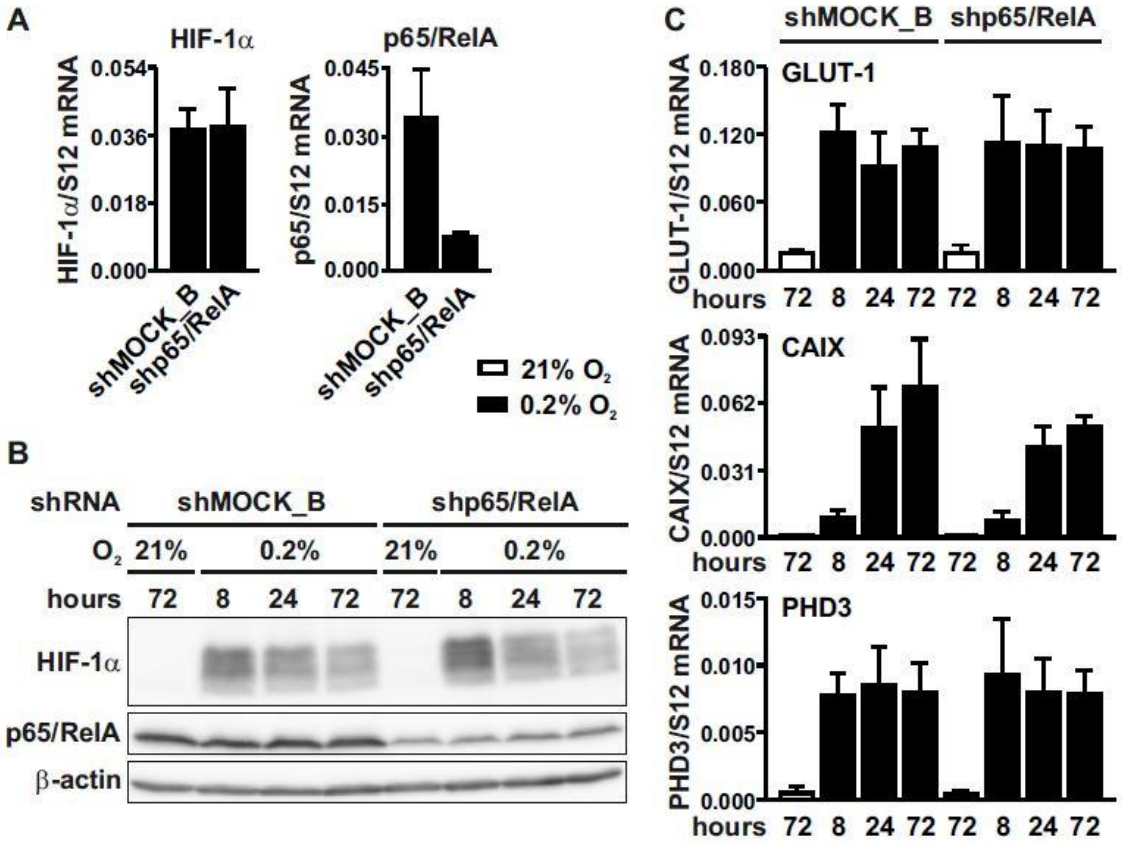

A
HIF-1α
HIF-1α/S12 mRNA
0.054
0.036
0.018
0.000
shMOCK_B
shp65/RelA
p65/RelA
p65/S12 mRNA
0.045
0.030
0.015
0.000
shMOCK_B
shp65/RelA
21% O2
0.2% O2
B
shRNA shMOCK_B shp65/RelA
O2 21% 0.2% 21% 0.2%
hours 72 8 24 72 72 8 24 72
HIF-1α
p65/RelA
β-actin
C
shMOCK_B shp65/RelA
GLUT-1
GLUT-1/S12 mRNA
0.180
0.120
0.060
0.000
hours 72 8 24 72 72 8 24 72
CAIX
CAIX/S12 mRNA
0.093
0.062
0.031
0.000
hours 72 8 24 72 72 8 24 72
PHD3
PHD3/S12 mRNA
0.015
0.010
0.005
0.000
hours 72 8 24 72 72 8 24 72

Fig.6

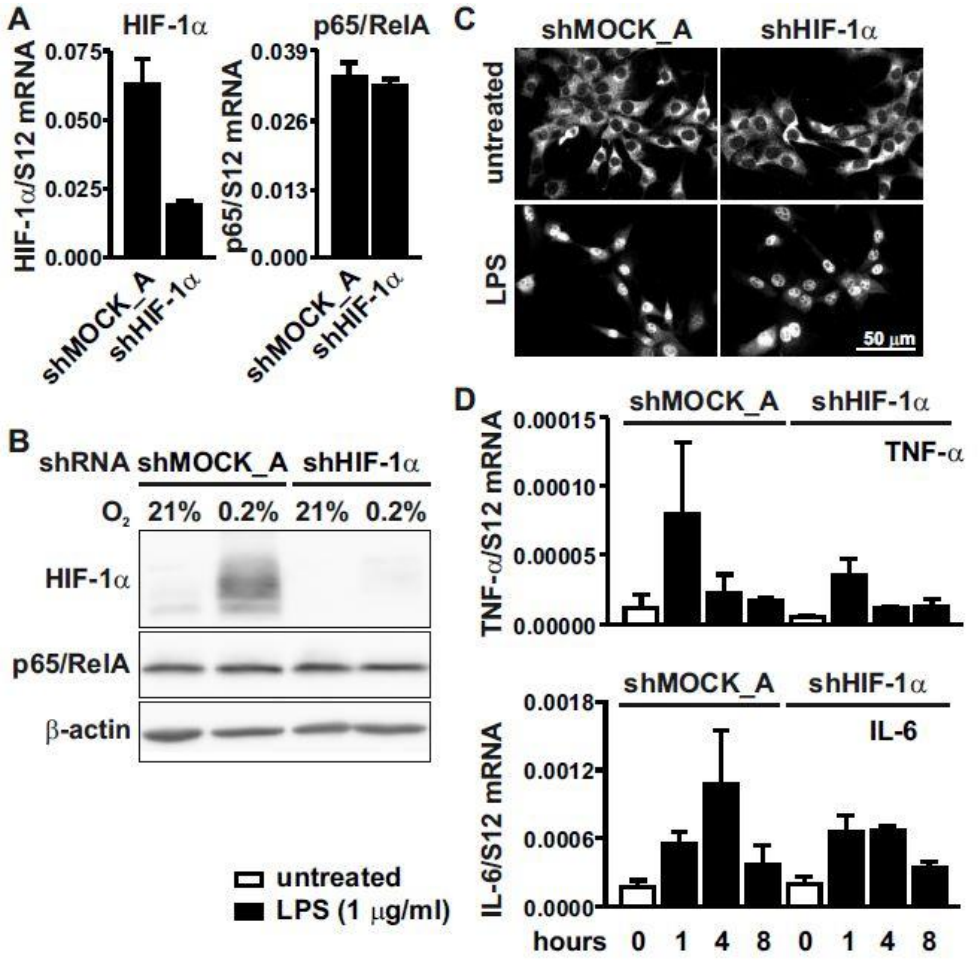

A
HIF-1α
p65/RelA
HIF-1α/S12 mRNA
p65/S12 mRNA
0.075
0.050
0.025
0.000
0.039
0.026
0.013
0.000
shMOCK_A
shHIF-1α
B
shRNA shMOCK_A shHIF-1α
O2 21% 0.2% 21% 0.2%
HIF-1α
p65/RelA
β-actin
untreated
LPS (1 μg/ml)
C
shMOCK_A
shHIF-1α
untreated
LPS
50 μm
D
shMOCK_A shHIF-1α
TNF-α
TNF-α/S12 mRNA
0.00015
0.00010
0.00005
0.00000
shMOCK_A shHIF-1α
IL-6
IL-6/S12 mRNA
0.0018
0.0012
0.0006
0.0000
hours 0 1 4 8 0 1 4 8

Fig.7

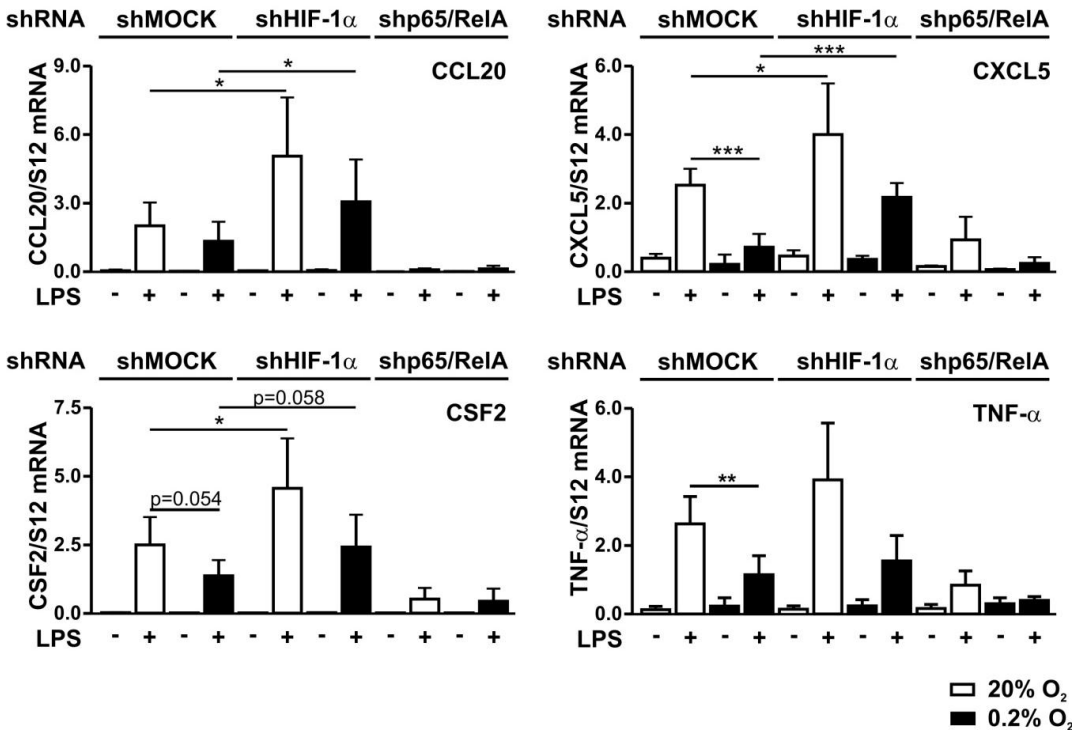

shRNA shMOCK shHIF-1α shp65/RelA
CCL20
CCL20/S12 mRNA
9.0
6.0
3.0
0.0
LPS - + - + - + - + - + - +
CXCL5
CXCL5/S12 mRNA
6.0
4.0
2.0
0.0
***
CSF2
CSF2/S12 mRNA
7.5
5.0
2.5
0.0
p=0.058
p=0.054
TNF-α
TNF-α/S12 mRNA
**
20% O2
0.2% O2

Fig.8

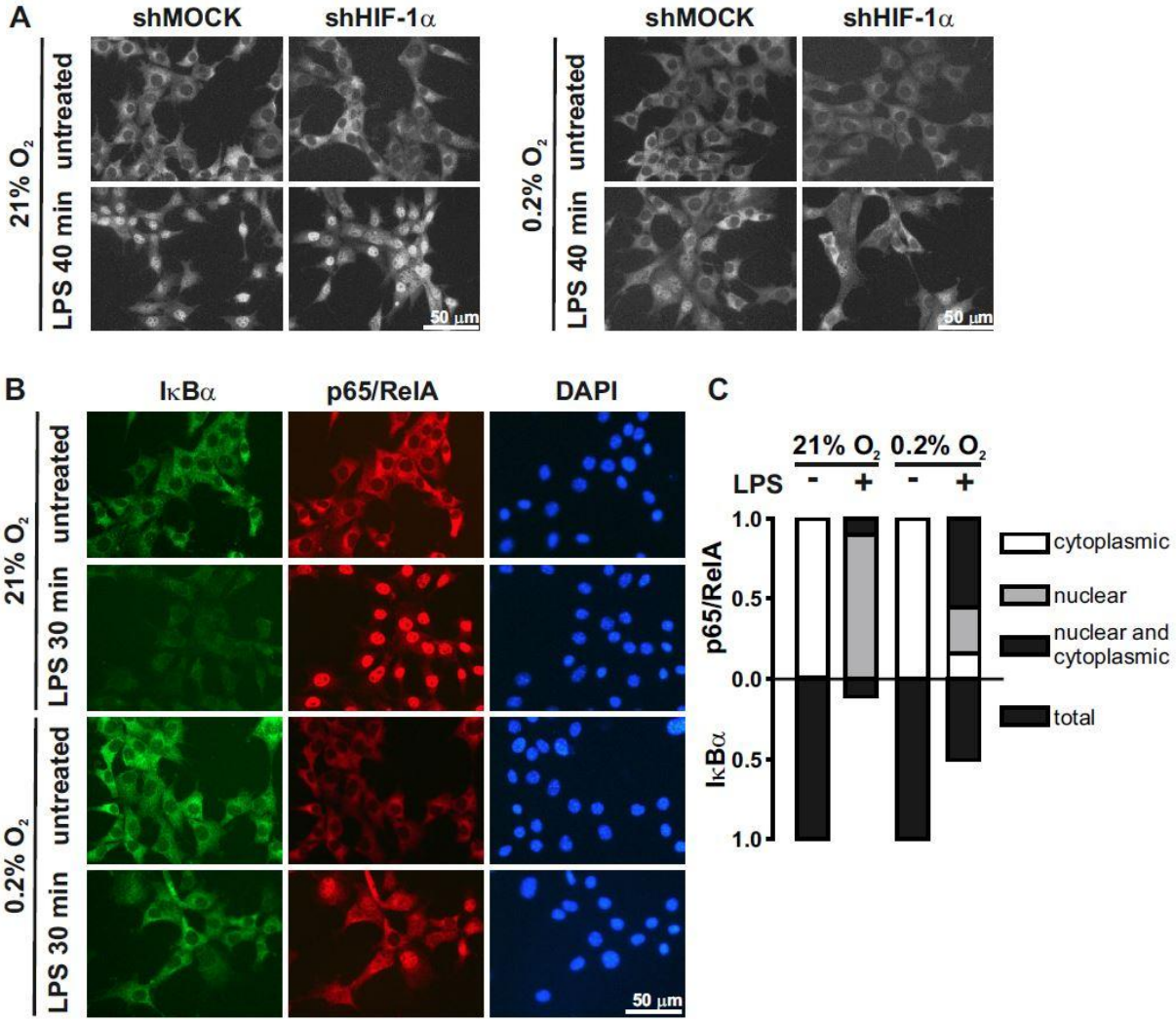

A
shMOCK
shHIF-1α
shMOCK
shHIF-1α
21% O2
untreated
LPS 40 min
0.2% O2
untreated
LPS 40 min
50 μm
50 μm
B
IκBα
p65/RelA
DAPI
21% O2
untreated
LPS 30 min
0.2% O2
untreated
LPS 30 min
50 μm
C
21% O2
0.2% O2
LPS
-
+
-
+
p65/RelA
1.0
0.5
0.0
IκBα
0.5
1.0
cytoplasmic
nuclear
nuclear and cytoplasmic
total

Fig.S1

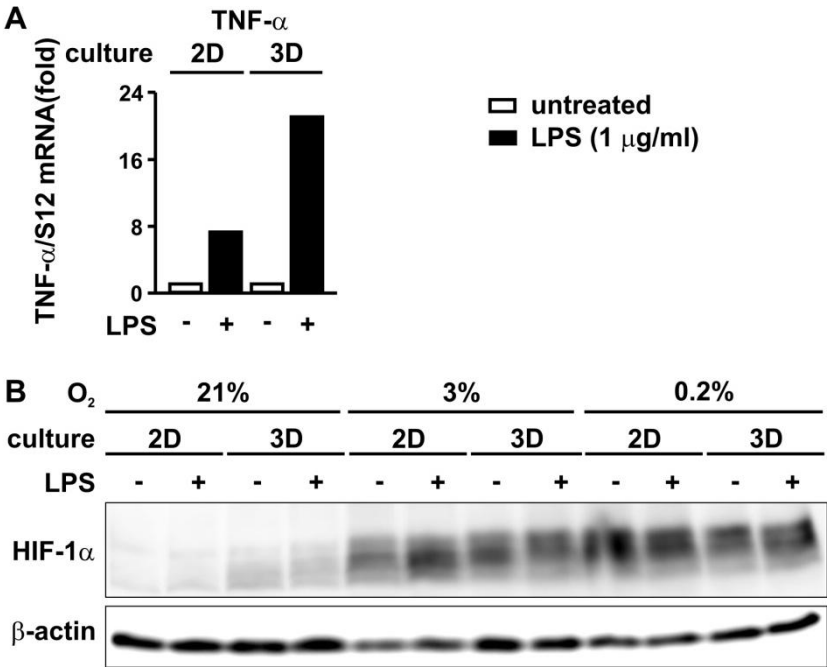

A
TNF-α
culture
2D
3D
TNF-α/S12 mRNA(fold)
24
16
8
0
LPS - + - +
untreated
LPS (1 μg/ml)
B
O2
21%
3%
0.2%
culture
2D
3D
2D
3D
2D
3D
LPS - + - + - + - + - + - +
HIF-1α
β-actin

Fig.S2

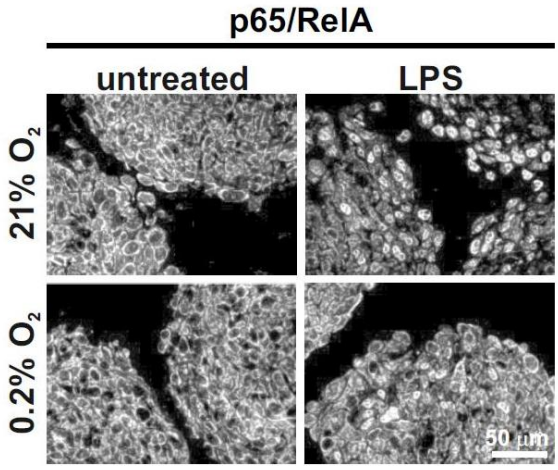

p65/RelA
untreated
LPS
21% O2
0.2% O2
50 μm

Fig.S3

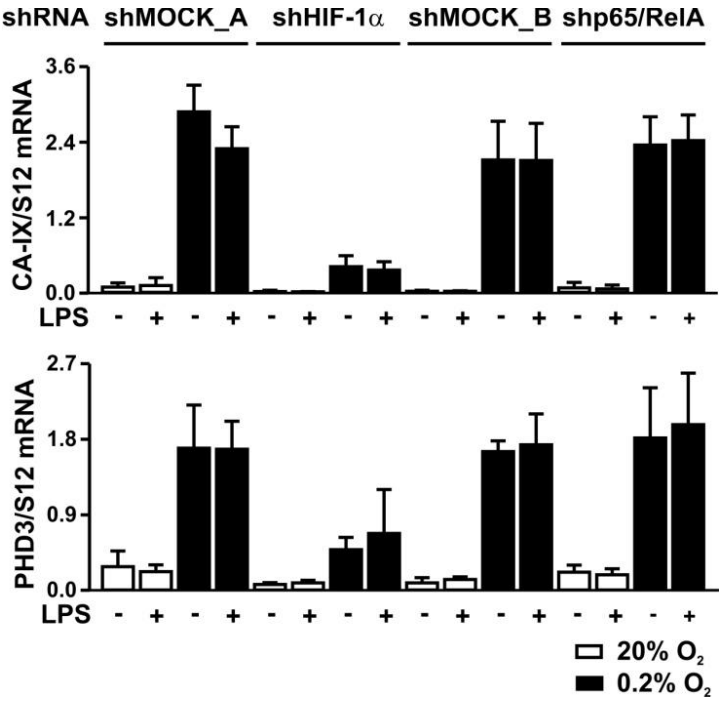
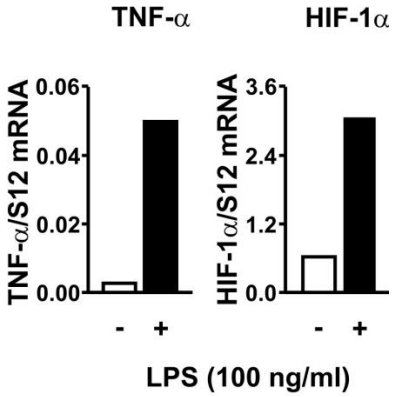

shRNA
shMOCK_A
shHIF-1α
shMOCK_B
shp65/RelA
CA-IX/S12 mRNA
3.6
2.4
1.2
0.0
LPS
- + - + - + - + - + - + - + - +
PHD3/S12 mRNA
2.7
1.8
0.9
0.0
LPS
- + - + - + - + - + - + - + - +
20% O2
0.2% O2

Fig.S4


TNF-α
HIF-1α
TNF-α/S12 mRNA
0.06
0.04
0.02
0.00
HIF-1α/S12 mRNA
3.6
2.4
1.2
0.0
- +
- +
LPS (100 ng/ml)

Tab. S1

Gene regulation revealed by RNA sequencing

Conditions	**+Hyp/-LPS vs. -Hyp/-LPS**	**+Hyp/+LPS vs. -Hyp/-LPS**	**-Hyp/+LPS vs. -Hyp/-LPS**	**+Hyp/+LPS vs. +Hyp/-LPS**
upregulated genes (total)	924	645	277	141
regulated by:				
HIF-1α (+)	663	393	45	26
HIF-1α (-)	1	7	6	9
HIF-1α (total)	664	400	51	35
p65/RelA (+)	708	135	164	34
p65/RelA (-)	2	4	0	4
p65/RelA (total)	710	139	164	38
HIF-1α (+), p65/RelA (+)	538	77	31	11
HIF-1α (-), p65/RelA (-)	0	0	0	1
HIF-1α (+), p65/RelA (-)	1	4	0	0
HIF-1α (-), p65/RelA (+)	0	4	2	5
HIF-1α and p65/RelA (total)	539	85	33	17
downregulated genes (total)	429	332	36	105
regulated by:				
HIF-1α (+)	12	12	0	2
HIF-1α (-)	106	78	17	53
HIF-1α (total)	118	90	17	55
p65/RelA (+)	155	10	0	2
p65/RelA (-)	50	60	21	76
p65/RelA (total)	205	70	21	78
HIF-1α (+), p65/RelA (+)	6	3	0	0
HIF-1α (-), p65/RelA (-)	16	35	9	36
HIF-1α (+), p65/RelA (-)	0	0	0	2
HIF-1α (-), p65/RelA (+)	32	0	0	1
HIF-1α and p65/RelA (total)	54	38	9	39

Tab. S2

Primers used for real-time PCR

mRNA		forward primer	reverse primer
CAIX	carbonic anhydrase 9	5’-gctgtcccatttggaagaaa-3’	5’-ggaaggaagcctcaatcgtt-3’
CCL20	chemokine (C-C motif) ligand 20	5’-cgactgttgcctctcgtaca-3’	5’-gaggaggttcacagcccttt-3’
COX-2	cytochrome oxidase 2	5’-tcctcctggaacatggactc-3’	5’-ccccaaagatagcatctgga-3’
CSF2	colony stimulating factor 2	5’-acatgcctgtcacgttgaat-3’	5’-ttgagtttggtgaaattgcc-3’
CXCL5	chemokine (C-X-C motif) ligand 5	5’-gctgctgtgtcatgcagaaaccta-3’	5’-gacattatgccatactacgaagacatc-3’
GLUT-1	glucose transporter 1	5’-tctctgtcggcctctttgtt-3’	5’-gcagaagggcaacaggatac-3’
HIF-1α	hypoxia-inducible factor 1α	5’-acaagtcaccacaggacag-3’	5’-agggagaaaatcaagtcg-3’
HIF-2α	hypoxia-inducible factor 2α	5’-taaagcggcagctggagtat-3’	5’-actgggaggcatagcactgt-3’
IL-6	interleukin 6	5’-tcctctctgcaagagacttccatcc-3’	5’-aagcctccgacttgtgaagtggt-3’
p65/RelA	NF-κB subunit p65	5’-gcgtacacattctggggagt-3’	5’-accgaagcaggagctatcaa-3’
PHD3	prolyl-4-hydroxylase domain protein 3	5’-caacttcctcctgtccctca-3’	5’-ggctggacttcatgtggatt-3’
S12	ribosomal protein S12	5’-gaagctgccaaagccttaga-3’	5’-aactgcaaccaaccaccttc-3’
TNF-α	tumour necrosis factor α	5’-gtcgtagcaaaccaccaagtgg-3’	5’-gagatagcaaatcggctgacgg-3’

6.2. Additional unpublished data

RESULTS

Expression of HIF-1α mRNA following LPS exposure is independent on DNA methylation in MC-38 cells

Our previous data revealed no transcriptional regulation of *Hif1a* in MC-38 cells, following NF-κB activating LPS treatment (see chapter 6.1.). However, binding of NF-κB to DNA can be inhibited in a methylation-dependent manner (*311, 312*). In addition, methylation of *Hif1a* gene was shown to be involved in regulation of HIF-1α expression in colon cancer cells (*313*). Therefore, MC-38 cells were treated with DNA demethylating agent 5-aza-2´-deoxycytidine (5-aza-2´-dC), to test the possible methylation of κB site in *Hif1a* gene as a reason for lack of NF-κB-mediated transcriptional HIF-1α upregulation in those cells. Neither the LPS treatment of conventional two-dimensional (2D) cultures, nor of spheroid 3D cultures (hanging drops) of MC-38 cells preexposed to 5-aza-2´-dC, affected the levels of HIF-1α transcript (FIG.1A-B). Importantly, mRNA levels of amphiregulin (AREG), whose expression was previously shown to be regulated by DNA methylation (*314*), was increased in cells treated with 5-aza-2´-dC. AREG transcript upregulation reached 7.1- and 4-fold in 2D and 3D, respectively (compare LPS- and 5-aza-2´-dC-untreated cells to LPS-untreated and 0.35 μM 5-aza-2´-dC-treated), indicating efficient DNA demethylation upon treatment of MC-38 cells with 5-aza-2´-dC (Figure 1A-B). Simultaneously, stimulation with LPS upregulated a known NF-κB target gene *Tnfa* expression in 2D as well as in 3D MC-38 cell cultures, indicating that NF-κB pathway was induced in these experimental conditions (Figure 1A-B). Despite induction of AREG mRNA expression (demethylation treatment positive control) and induction of TNF-α (LPS treatment positive control) the levels of HIF-1α transcript remained unaltered following the exposure of cells to proinflammatory treatment, excluding inhibitory effect of methylation of κB site in *Hif1a* locus as a reason for lack of its transcriptional regulation by NF-κB in MC-38 cells.

Knock-down of p65/RelA does not influence HIF-1α protein accumulation in hypoxic MC-38 cells

To confirm our previous findings on no effect of p65/RelA knock-down on HIF-1α accumulation in hypoxia (see chapter 6.1.), two independent shRNA-mediated p65/RelA knock-downs in MC-38 cells were established. Accumulation of HIF-1α protein in hypoxia, in both shp65/RelA#1 and shp65/RelA#2 MC-38 cell lines remained unaltered compared to shMOCK_A control cells (Figure 2). Expression of HIF-1α protein in hypoxia is independent of the basal expression levels of p65/RelA in MC-38 cells.

shRNA-mediated knock-down of HIF-1α in MC-38 cells grown in 3D cultures displays a robust decrease in HIF-1α expression

To confirm the high efficiency of stable HIF-1α knock-down in MC-38 cells in 3D spheroid cell culture conditions, shHIF-1α, shp65/RelA and shMOCK control cell lines (see Chapter 6.1.) were cultivated in 3D hanging drops for 48 h, followed by the exposure to hypoxia and/or LPS. Efficient knock-down of HIF-1α in shHIF-1α compared to shMOCK_A cells could be confirmed on the mRNA level (81% reduction in LPS-untreated in normoxia). Furthermore, HIF-1α transcript abundance was unaffected by shp65/RelA expression in MC-38 cells (Figure 3A). Knock-down of HIF-1α could be further evidenced on the protein levels in shHIF-1α MC-38 cells grown as for Figure 3A and subsequently exposed to hypoxia or the PHD enzyme inhibitor DMOG (Figure 3B).

shRNA-mediated knock-down of p65/RelA in MC-38 cells grown in 3D cultures displays a robust decrease in p65/RelA expression

To confirm the high efficiency of stable p65/RelA knock-down in MC-38 cells in 3D spheroid culture conditions, shp65/RelA, shHIF-1α and shMOCK control cell lines (see Chapter 6.1.) were cultivated and treated as for Figure 3A. Efficient knock-down of p65/RelA in shp65/RelA compared to shMOCK_B cells could be confirmed on the mRNA levels (64% reduction in LPS-untreated in normoxia). Moreover, p65/RelA transcript abundance was unaffected by shHIF-1α expression in MC-38 cells (Figure 4A). Knock-down of p65/RelA could be also shown on the protein levels in shp65/RelA MC-38 cells grown and treated as for Figure 3A (Figure 4B).

LPS-mediated response is attenuated by HIF-1α in conventional 2D MC-38 cell cultures

Our previous results, showed that the response to treatment with LPS under hypoxia is decreased compared to normal oxygen conditions in 3D spheroid cultures of MC-38 cells (see chapter 6.1.). For a subset of well known NF-κB target genes including *Ccl20*, *Cxcl5*, *Csf2* and *Tnfa,* HIF-1α was found to be a negative regulator of their LPS-mediated induction (see chapter 6.1.). To confirm these findings in conventional 2D cultures, shMOCK_A and shHIF-1α MC-38 cells were exposed to hypoxia and/or LPS. CXCL5, CSF2 and TNF-α mRNA levels in control shMOCK_A cells were 4.6-, 1.9-, 2.9-, respectively, higher following the LPS treatment in normoxia than in hypoxia. However, CCL20 mRNA displayed no hypoxic effect on its LPS-response inducibility. In the case of CXCL5, CSF2 and TNF-α transcripts, HIF-1α knock-down at least partially rescued the hypoxic repression of their response to LPS treatment. Interestingly, HIF-1α knock-down cells showed increased CCL20 mRNA response to LPS compared to control cells irrespective of the oxygen conditions. The LPS-induced increase in CCL20 transcript abundance in shHIF-1α compared to shMOCK_A cells reached 2.4- and 4.2-fold in normoxia and hypoxia, respectively. A summary of these findings is displayed in the Figure 5.

DISCUSSION

Direct regulation of HIF-1α mRNA expression by NF-κB has been described before in several models including different macrophage cell lines and primary human macrophages, as well as human embryonic kidney (HEK) 293, and pulmonary artery smooth muscle cells (PASMCs) (*264-268*). Furthermore, in the absence of functional NF-κB pathway, hypoxic activation of HIF-1α expression in bone marrow-derived macrophages (BMDMs) as well as livers and brains of mice was decreased (*263*). However, our previous data indicated no transcriptional regulation of HIF-1α mRNA expression in MC-38 cells stimulated with LPS despite robust concomitant NF-κB activation. Consistently, we could not observe attenuated hypoxic HIF-1α activation in p65/RelA knock-down MC-38 cells (see chapter 6.1.). As one of the possible mechanisms why this process may be limited only to particular cells, methylation of the κB site in the *Hif1a* gene was considered. Methylation of the NF-κB binding DNA sites was previously suggested to be involved in the regulation of the expression of

known NF-κB target gene iNOS as well as binding of NF-κB to cognate motifs in HIV-1 long terminal repeats (*311, 312*). In addition, HIF-1α transcription induction in hypoxia has been shown to be dependent on the lack of HRE methylation and therefore autoregulatory HIF-1α binding in colon cancer cell lines, indicating the involvement of DNA methylation in the HIF-1α mRNA expression (*313*). To test the possible inhibitory effect of methylation of κB site on the NF-κB binding to *Hif1a* promoter and therefore attenuation of NF-κB-mediated HIF-1α induction in MC-38 cells, the treatment with demethylating agent 5-aza-2´-deoxycytidine (5-aza-2`-dC) was applied. One of the mechanisms by which 5-aza-2`-dC mediates DNA demethylation is its incorporation to DNA and subsequent covalent binding-mediated inactivation of the DNA methyltransferases (*315, 316*). However, the pretreatment of MC-38 cells with 5-aza-2`-dC did not result in the increase in HIF-1α transcript abundance following the proinflammatory stimulation, ruling out methylation of the κB site in *Hif1a* promoter as a mechanism of lack of NF-κB-mediated HIF-1α induction in MC-38 cells. In agreement with no LPS-mediated regulation of HIF-1α transcription in MC-38 cells, we previously showed no alterations in hypoxic HIF-1α protein accumulation in cells expressing stable shRNA targeting p65/RelA (see chapter 6.1.). In the current work, these findings were confirmed in two independent shp65/RelA MC-38 cell lines, further supporting no dependence of HIF-1α expression on NF-κB in MC-38 cells. The direct regulation of HIF-1α mRNA following the proinflammatory treatment remains a compelling target to study the interplay between hypoxia and inflammation. The transcriptional induction of HIF-1α by NF-κB appears to be cell type specific. Among proposed mechanisms of the specificity of NF-κB-mediated transcription activation in distinct cell types is differential recruitment of transcriptional cofactors e.g. MYB-binding protein 1A (MYBBP1A) and downregulated in metastasis (DRIM), both enriched in complexes with TAD domain of p65/RelA in nuclear extracts from Jurkat cells, IQ motif containing GTPase activating protein 3 (IQGAP3) in THP-1, or human general control of amino acid synthesis 1-like 1 (hGCN1L1) in HeLa (*317*). However, the factors responsible for cell-type specific regulation of HIF-1α transcript abundance following activation of NF-κB are currently unknown.

Our previous data suggested that hypoxia attenuates the cellular response to LPS, and that HIF-1α is a negative regulator of a panel of known NF-κB target genes

Ccl20, *Cxcl5*, *Csf2* and *Tnfa*, involved in the recruitment and activation of leucocytes (see chapter 6.1.). Mentioned results were obtained in MC-38 cells grown in 3D spheroid cultures, which display more robust activation following proinflammatory stimulation with LPS compared to cells cultivated in commonly applied 2D cell growth conditions in tissue culture dishes (see chapter 6.1.). In the current work, the findings on the negative regulation of LPS-stimulated expression of CCL20, CXCL5, CSF-2 and TNF-α mRNAs by HIF-1α could also be confirmed in 2D cell cultures, suggesting that this effect is not restricted to hanging drop MC-38 cultures.

It was previously shown that hypoxia and in particular HIF-1α is involved in the $CD45^+$ myeloid cell recruitment from the bone marrow in glioblastoma, an effect at least partially dependent on HIF-1α-dependent induction of CXCL12 chemokine (also known as stromal cell-derived factor 1 - SDF-1) (*140*). However, by attenuation of the LPS-mediated upregulation of CCL20, CXCL5, CSF-2 and TNF-α expression, HIF-1α may possibly play another context-dependent role, in regulating the inflammatory component in the colon cancer. Follow-up studies should aim at verifying the inhibitory role of HIF-1α on the expression of those inflammatory mediators in mouse colon tumors, as well as the downstream effects of this interaction axis on the inflammatory composition of tumor microenvironment.

MATERIALS AND METHODS

5-aza-2´-deoxycytidine (5-aza-2´-dC) was purchased from Sigma (St. Louis, MO, USA). Knock-down of specific mRNAs was achieved by lentiviral transduction of short hairpin RNA (shRNA) vectors driven by the U6 promoter in the pLKO.1-puro plasmid (Sigma). Vectors targeting mouse p65/RelA (shp65/RelA#1 and shp65/RelA#2) and non-target controls (shMOCK_A) were purchased from Sigma. Primers used for RT-qPCR were: mouse β-actin forward 5'-gagcgtggctacagcttcac-3' and reverse 5'-ggcatagaggtctttacggatg-3' and mouse AREG forward 5'-gactcacagcgaggatgaca-3' and reverse 5'-ggcttggcaatgattcaact-3'. Remaining materials and methods were described in the chapter 6.1.

FIGURE LEGENDS

Figure 1. 5-aza-2´-dC treatment of MC-38 cells. RT-qPCR analysis of the abundance of AREG, TNF-α and HIF-1α transcripts in MC-38 cells pretreated with 0.25 or 0.35 μM 5-aza-2´-dC for 96 h. **(A)** Cells were starved for 16 h in medium containing 0.1 %

FCS, followed by the exposure to 1 μg/ml LPS for 1 h. **(B)** Cells were cultivated in 3D spheroids for 24 h, followed by the stimulation with 1 μg/ml LPS for 1 h. Data were normalized to the constitutively expressed β-actin mRNA levels.

Figure 2. Hypoxic HIF-1α accumulation in cells stably expressing shRNA targeting p65/RelA in MC-38 cells. Immunoblot detection of HIF-1α and p65/RelA in 50 μg of total extracts derived from stable shRNA-mediated knock-down shp65/RelA#1, shp65/RelA#2, shHIF-1α and control shMOCK_A MC-38 cell lines exposed to hypoxia (0.2 % oxygen) for 8 h. β-actin served as a loading control.

Figure 3. Stable shRNA-mediated HIF-1α knock-down in MC-38 cells cultured in 3D spheroids. shHIF-1α and shMOCK_A, as well as shp65/RelA and shMOCK_B MC-38 cells were grown for 48 h in 3D spheroid cultures and exposed to hypoxia (0.2% oxygen) or 1 mM DMOG for 8 h, followed by the treatment with 1 μg/ml LPS for the last 1 h of hypoxic/DMOG cell cultures. **(A)** HIF-1α mRNA levels were quantified by RT-qPCR. Data were normalized to the constitutively expressed ribosomal protein S12 mRNA. **(B)** HIF-1α protein levels were analysed by immunoblotting of 50 μg of total protein extracts. β-actin served as loading control.

Figure 4. Stable shRNA-mediated p65/RelA knock-down in MC-38 cells cultured in 3D. shHIF-1α and shMOCK_A, as well as shp65/RelA and shMOCK_B MC-38 cells were grown for 48 h in 3D spheroid cultures and exposed to hypoxia (0.2% oxygen) for 8 h, followed by the treatment with LPS for the last 1 h of hypoxic or normoxic cell cultures. **(A)** p65/RelA mRNA levels were quantified by RT-qPCR. Data were normalized to the constitutively expressed ribosomal protein S12 mRNA. **(B)** p65/RelA protein levels were analysed by immunoblotting of 50 μg total protein extracts. β-actin served as loading control.

Figure 5. The effect of HIF-1α on the expression of known NF-κB target genes in MC-38 cultivated in conventional 2D cultures. CCL20, CXCL5, CSF2 and TNF-α mRNA levels were quantified by RT-qPCR in 2D MC-38 cultures prestarved for 16 h in medium containing 0.1% FCS, exposed to hypoxia (0.2% oxygen) for 8 h and treated with 1 μg/ml LPS for the last 1 h of hypoxic or normoxic exposure. Data were normalized to the constitutively expressed ribosomal protein S12 mRNA levels.

FIGURES

Fig.1

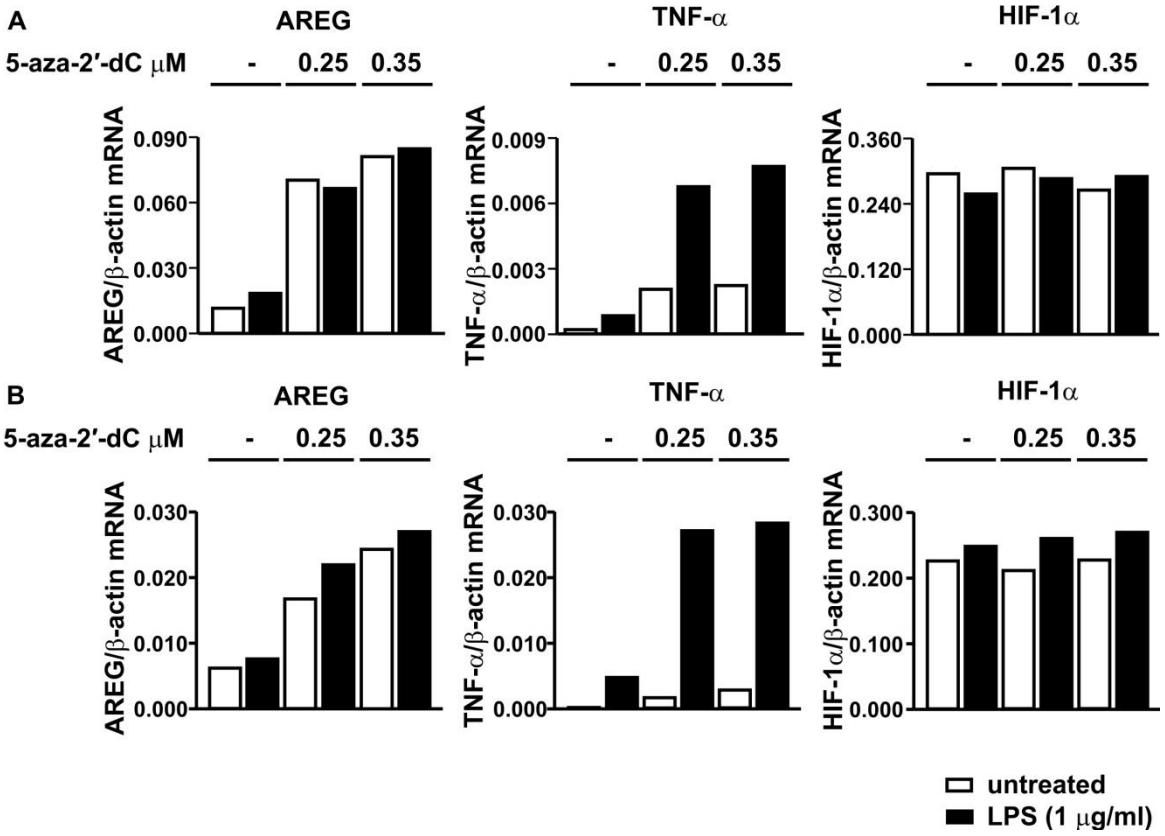


Fig.2

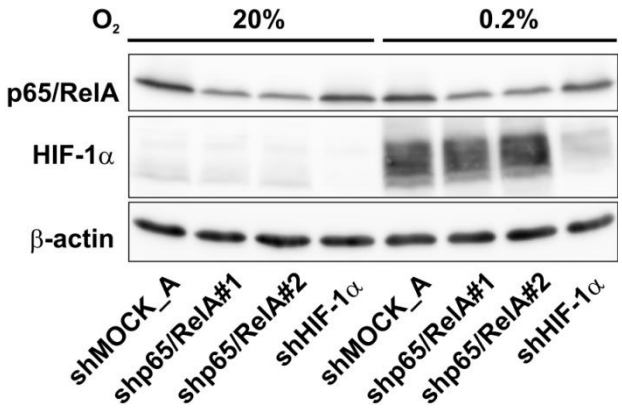
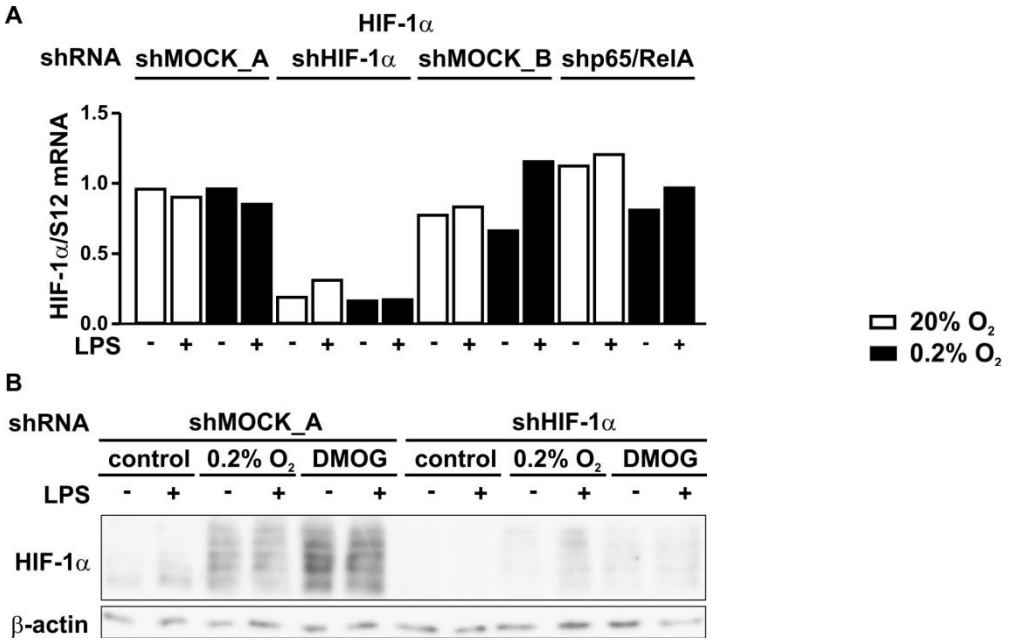


Fig.3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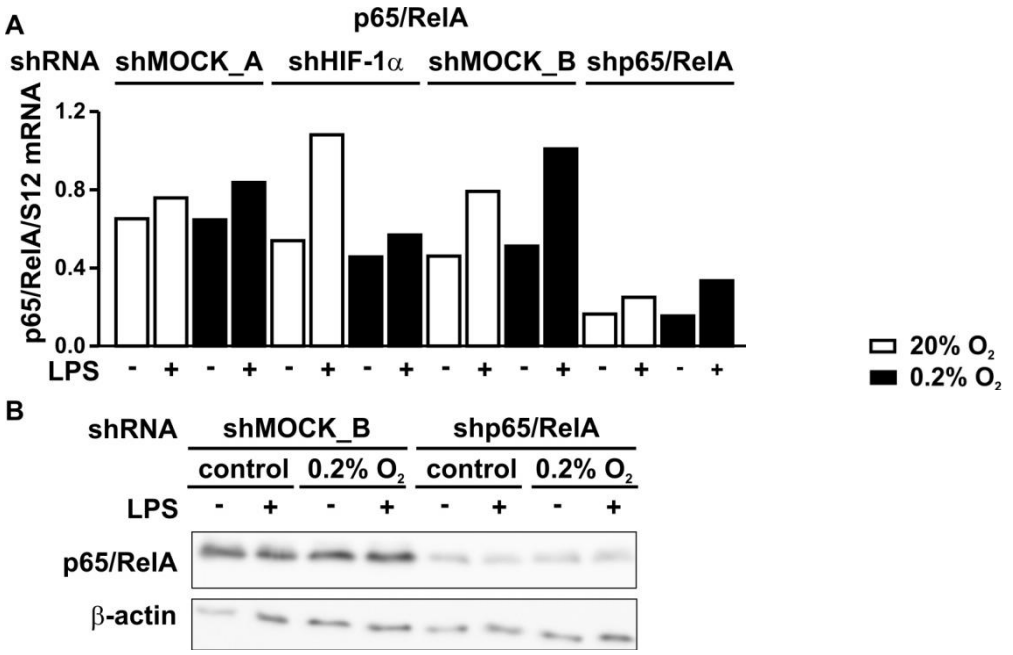
HIF-1α
shRNA
shMOCK_A
shHIF-1α
shMOCK_B
shp65/RelA
HIF-1α/S12 mRNA
1.5
1.0
0.5
0.0
LPS
- + - + - + - + - + - + - + - +
20% O2
0.2% O2
B
shRNA
shMOCK_A
shHIF-1α
control
0.2% O2
DMOG
control
0.2% O2
DMOG
LPS
- + - + - + - + - + - + - +
HIF-1α
β-actin

Fig.4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shMOCK_A
shHIF-1α
shMOCK_B
shp65/RelA
p65/RelA/S12 mRNA
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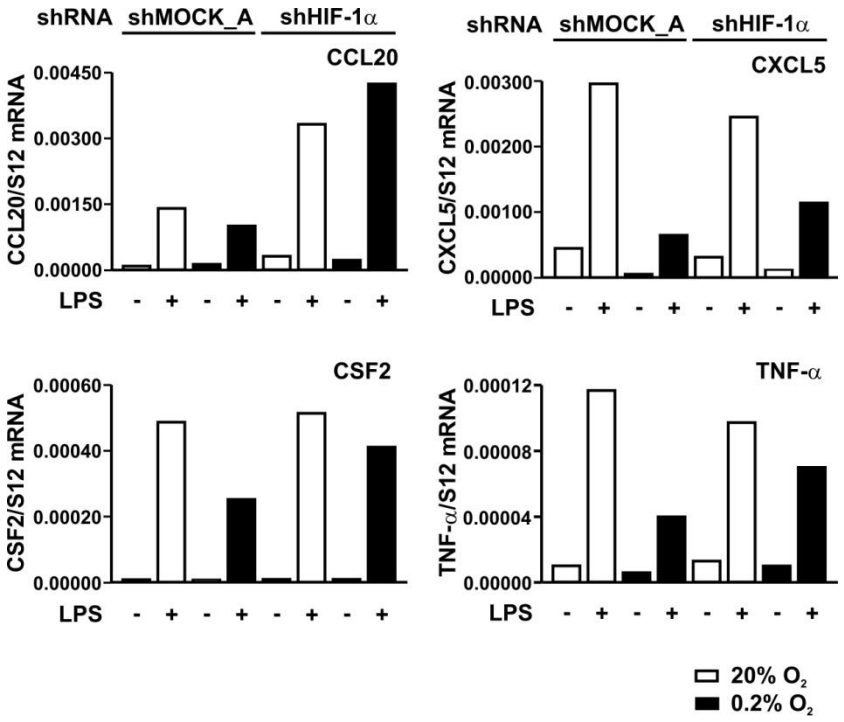
0.8
0.4
0.0
LPS
- + - + - + - + - + - + - + - +
20% O2
0.2% O2
B
shRNA
shMOCK_B
shp65/RelA
control
0.2% O2
control
0.2% O2
LPS
- + - + - + - +
p65/RelA
β-actin

Fig.5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CCL20
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0.00000
LPS - + - + - + - +
CXCL5
CXCL5/S12 mRNA
0.00300
0.00200
0.00100
0.00000
CSF2
CSF2/S12 mRNA
0.00060
0.00040
0.00020
0.00000
TNF-α
TNF-α/S12 mRNA
0.00012
0.00008
0.00004
0.00000
20% O2
0.2% O2

6.3. Personal contribution

- **Hypoxia attenuates the inflammatory response in colon cancer cells by affecting the levels of IκB. Kamila Bartnicka[1,2,6], Karolin Léger[2,3,6], Jesus F. Glaus Garzon[1,2], Carole Oertli[1,2], Ali Mirsaidi[2,3], Peter J. Richards[4], Hubert Rehrauer[5], Patrick Spielmann[1,2], David Hoogewijs[1,2], Lubor Borsig[1,2,7], Michael Hottiger[2,3,7], Roland H. Wenger[1,2,7,*]**

  Concerning performing the experiments:
  Fig.1 – all
  Fig.2 – all, except 2A (equally contributed with KL)
  Fig.3 – all
  Fig.4 – all
  Fig.5 – all, except generating shp65/RelA MC-38 cells
  Fig.6 – all, except 6C (equally contributed with KL)
  Fig.7 – all but equally contributed with KL
  Fig.8 – 8A (equally contributed with KL), 8B (equally contributed with PS), 8C – all
  Fig.S1 – all but equally contributed with KL
  Fig.S2 – all but equally contributed with KL and AM
  Fig.S3 – all but equally contributed with KL
  Fig.S4 – all
  Tab.S1 – all but equally contributed with KL

- **Additional unpublished data.**

  Concerning performing the experiments:
  Fig.1 – all
  Fig.2 – all
  Fig.3 – all but equally contributed with KL
  Fig.4 – all but equally contributed with KL
  Fig.5 – all

7. Conclusions

Colorectal cancer (CRC) represents an interesting model to study the interplay between hypoxic and inflammatory pathways. It is an example of a malignancy with well established link to chronic inflammation, such as in inflammatory bowel disease (*208, 209*). CRCs are commonly characterized by the presence of hypoxia and inflammation, and activation of HIF-1α as well as NF-κB in this type of malignancy implies worse patient prognosis (*162, 214, 251, 258, 259*).

Mouse colon adenocarcinoma MC-38 cells were employed to investigate the cross-talk between hypoxia and inflammation in colon cancer. The response to hypoxia in MC-38 cells is mediated by HIF-1. Consistently, HIF-1α protein and its classical target genes (*Glut1*, *Ca9* and *Phd3*) were strongly induced upon hypoxic exposure of MC-38 cells.

It was shown before, that the germ-free rats developed fewer and smaller chemically-induced tumours compared to animals carrying regular gut microflora, suggesting a role of commensal microorganisms in colorectal carcinogenesis (*253*). The most common colonic bacteria are Gram-negative species whose outer membrane component lipopolysaccharide (LPS) is recognized by TLR4 (*254*). TLR4 knock-out mice were protected from tumours in a model of AOM/DSS-induced colon carcinogenesis, indicating a role of TLR4 signalling in CRC (*255*). Importantly, components of LPS signalling, TLR4 receptor and adapter MyD88, were shown to be overexpressed in tumour cells compared to normal and adenomatous epithelium in sporadic human colon cancers, and expression of TLR4 and MyD88 was associated with poor patient prognosis (*256*). Similarly, majority of colitis-associated cancers (CACs) were shown to display high expression levels of epithelial TLR4 (*257*), further underscoring the importance of TLR4 signalling in colon cancer cells. Therefore, in the current work, LPS was applied as a stimulus of inflammatory response of colon cancer cells.

MC-38 cells displayd robust response of the transcription factor NF-κB to proinflammatory treatment with LPS. The strong response of MC-38 cells to LPS was evidenced by nuclear translocation of NF-κB upon exposure to proinflammatory stimulation. In addition, classical NF-κB target gene (*Tnfa*, *Il6* and *Cox2*) expression

was upregulated under the same conditions, confirming NF-κB activation in MC-38 cells subjected to treatment with LPS. MC-38 cells represent a suitable model to study the interplay between hypoxia and inflammation in this cancer type.

It has been previously shown, that exposure to hypoxia may activate NF-κB (*262, 274-276*). Although MC-38 cells respond to hypoxia by upregulating the HIF pathway, hypoxic treatment does not lead to an increase in NF-κB activation. No nuclear accumulation of NF-κB was observed in MC-38 cells exposed to hypoxia. Consistently, hypoxic exposure did not alter the mRNA levels of classical NF-κB target genes *Tnfa*, *Il6* and *Cox2*. Hypoxia-mediated NF-κB activation does not occur in the MC-38 colon cancer cells and these results support the notion that increased activity of NF-κB in hypoxic conditions is not a ubiquitous phenomenon.

One of the levels of the putative cross-talk between hypoxic and inflammatory pathways is direct transcriptional regulation of HIF-1α mRNA by NF-κB that has been evidenced in different cells, but mainly macrophages (*263-268*). Although in the current work HIF-1α mRNA was shown to be regulated by NF-κB in mouse primary peritoneal macrophages, *Hif1a* gene is not a transcriptional target of NF-κB in MC-38 cells. Neither the transcript, nor the protein levels of HIF-1α were affected by proinflammatory treatment of those cells. Consistently, expression of the classical HIF target genes *Glut1*, *Ca9* and *Phd3* was unaltered over a time-course of proinflammatory LPS treatment in MC-38. In line with the findings in MC-38 cells, in a panel of human colon cancer cell lines, the treatment with LPS did not affect the levels of HIF-1α mRNA, suggesting that this observation may be characteristic for colorectal carcinoma cells.

Aiming at elucidating the mechanism responsible for lack of HIF-1α response to NF-κB activation in MC-38 cells, the possible methylation of κB site in *Hif1a* promoter was addressed. Previous reports showed that HIF-1α may upregulate its own expression in hypoxic colon cancer cells if the HRE in its gene is unmethylated (*313*). Moreover, it was evidenced before, that the methylation of κB sites may impair NF-κB binding (*311, 312*). To investigate the involvement of DNA methylation in the regulation of NF-κB-mediated *Hif1a* induction, MC-38 cells were exposed to demethylating agent 5-aza-2´-deoxycytidine (5-aza-2`-dC). 5-aza-2`-dC pretreatment

in MC-38 cells did not result in an increase in HIF-1α mRNA levels after proinflammatory stimulation with LPS, despite induction of amphiregulin (AREG) mRNA levels, whose expression was previously shown to be regulated by methylation (*314*) and served here as a positive control of the DNA demethylation. Therefore, methylation of κB site could not explain the lack of transcriptional activation of *Hif1a* gene by NF-κB in MC-38 cells. Other unknown factors must therefore be responsible for conferring the cell-type specificity of transcriptional HIF-1α response following proinflammatory treatment.

Basal activity of NF-κB pathway was shown to be required for HIF-1α response in hypoxic bone marrow-derived macrophages (BMDMs) as well as in livers and brains of mice (*263*). However, knock-down of p65/RelA neither resulted in alterations in HIF-1α mRNA levels, nor influenced HIF-1α protein accumulation or the response of its classical target genes (*Glut1*, *Ca9* and *Phd3*) in hypoxia. HIF-1α pathway activation in hypoxic MC-38 cell is independent of basal NF-κB activity.

To investigate whether basal activity of HIF-1α affects NF-κB signalling in MC-38 cells, the levels of p65/RelA mRNA and protein were tested in MC-38 stably expressing shRNA against HIF-1α. The levels of p65/RelA were not altered in HIF-1α knock-down compared to control cells. This result was further confirmed by the lack of influence of basal HIF-1α expression on the nuclear translocation of NF-κB or induction of well known NF-κB target genes (*Tnfa* and *Il6*) after treatment with LPS in MC-38 cells. Basal HIF-1α expression is therefore irrelevant for the NF-κB expression levels as well as for its activity following LPS treatment in MC-38 cells.

As another level of possible interactions between hypoxia and inflammation, the effect of combined simultaneous response was analysed. RNA deep sequencing analysis of the HIF-1α and/or p65/RelA-dependent gene regulation in combination with different oxygen and/or LPS treatment conditions, revealed surprisingly prominent levels of reciprocal dependence between both pathways. Almost two thirds of hypoxia-induced genes were influenced by expression of both, HIF-1α and NF-κB, whereas out of LPS-stimulated genes, regulation of about 12% was affected by expression of both transcription factors. Furthermore, the number of genes upregulated by the treatment with LPS in hypoxia was reduced by about 50% compared to proinflammatory stimulation under normal oxygen conditions.

Importantly, the lack of HIF-1α led to increased LPS-stimulated expression of several known NF-κB target genes. These findings were independently confirmed for CCL20, CXCL5, CSF2 and TNF-α transcript levels.

The following validation experiments revealed inhibition of the LPS-stimulated decrease in IκBα levels following the MC-38 cells treatment in hypoxic compared to normoxic conditions. As a result, nuclear p65/RelA translocation in hypoxia was attenuated and this observation at least in part explains the inhibitory effect of hypoxia on the response to LPS. However, nuclear translocation of p65/RelA in response to LPS treatment was comparably affected by hypoxia in control and HIF-1α knock-down cells. Therefore, further mechanistic insight into the role of HIF-1α in expression of *Ccl20*, *Cxcl5*, *Csf2* and *Tnfa* is required. An indirect mechanism (such as induction of microRNA(s) targeting those genes) should be considered, since the genome-wide analysis of HIF binding loci excluded transcriptional inhibition mediated by HIF association to DNA (*105*).

Hypoxia exerted inhibitory effects on the response of colon cancer cells to LPS treatment and HIF-1α itself had a negative effect on the expression of several strongly induced known NF-κB target genes. It was shown previously that HIF-1 has a protective role in mouse model of colitis, preserving the barrier function of affected epithelia (*260*). Current work shows that HIF-1 inhibits the expression of a set of important inflammatory mediators in response to LPS treatment in colon cancer cells, indicating the possible existence of another mode of HIF-1-mediatied negative regulation of inflammation by modulating the recruitment and activation of leucocytes in CRC.

Chronic intestinal inflammation is clearly associated with increased risk of development of CRC in humans (*208, 209*). Importantly, huge number of data on the possible use of nonsteroidal antiinflammatory drugs (NSAIDs) for chemoprevention of cancers has accumulated since late 1980s. NSAIDs inhibit cyclooxygenase 2 (COX-2) and therefore proinflammatory prostaglandin synthesis, and this is the most probable mechanism of their preventive action in cancer (*318*). Aspirin, which belongs to NSAIDs, has been independently by different investigators shown to reduce the risk of CRC (*319, 320*), further strongly supporting the importance of inflammation as a driving force of colonic carcinogenesis. In turn, animal experiments

contributed to the establishment of a role of permanently active NF-κB in the intestinal epithelium as a critical inducer of spontaneous tumourigensis (*207*). Current work shows that hypoxia which is a hallmark of virtually all solid tumours blunts the response of mouse colon adenocarcinoma MC-38 cells to proinflammatory treatment with LPS, which induces cancer cell signalling suggested before to be associated with worse CRC patient survival (*256*). The membrane-permeable dimethyloxalylglycine (DMOG) mimics hypoxia by inhibiting PHD enzymes competing with one of their substrate α-ketoglutarate (*321*). Interestingly, the treatment with DMOG was previously shown to suppress LPS-induced expression of cytokines in Raw264.7 macrophages (*278*). Our results support previously published data and suggest that the use of PHD enzyme inhibitors, such as DMOG, could be potentially beneficial in colorectal cancer patient treatment.

8. References

1. Ferlay J. *et al.* GLOBOCAN 2012 v1.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 11 [online]. International Agency for Research on Cancer, Lyon (France) (2013). Available from: http://globocan.iarc.fr.
2. Franks L. M., Knowles, M.A. What is cancer? in Introduction to the cellular and molecular biology of cancer, Oxford University Press (2005);
3. Willis R. A. The Spread of Tumors in the Human Body, Butterworth & Co. (1952);
4. Kumar V. *et al.* Robbins and Cotran pathologic basis of disease, Saunders (2005);
5. Morin R. D. *et al.* Frequent mutation of histone-modifying genes in non-Hodgkin lymphoma. Nature (2011);
6. van Haaften G. *et al.* Somatic mutations of the histone H3K27 demethylase gene UTX in human cancer. Nat Genet (2009);
7. Ley T. J. *et al.* DNMT3A mutations in acute myeloid leukemia. N Engl J Med (2010);
8. Jones S. *et al.* Frequent mutations of chromatin remodeling gene ARID1A in ovarian clear cell carcinoma. Science (2010);
9. Wang K. *et al.* Exome sequencing identifies frequent mutation of ARID1A in molecular subtypes of gastric cancer. Nat Genet (2011);
10. Khalil A. M. *et al.* Many human large intergenic noncoding RNAs associate with chromatin-modifying complexes and affect gene expression. Proc Natl Acad Sci U S A (2009);
11. Weston A., Harris,C.C. Chemical Carcinogenesis in Cancer Medicine, BC Decker (2003);
12. Knudson A. G., Jr. Mutation and cancer: statistical study of retinoblastoma. Proc Natl Acad Sci U S A (1971);
13. Friend S. H. *et al.* A human DNA segment with properties of the gene that predisposes to retinoblastoma and osteosarcoma. Nature (1986);
14. Dryja T. P. *et al.* Homozygosity of chromosome 13 in retinoblastoma. N Engl J Med (1984);
15. Nishizuka Y. The role of protein kinase C in cell surface signal transduction and tumour promotion. Nature (1984);
16. Li J. J. *et al.* Inhibitors of both nuclear factor-kappaB and activator protein-1 activation block the neoplastic transformation response. Cancer Res (1997);
17. Dong Z. *et al.* Blocking of tumor promoter-induced AP-1 activity inhibits induced transformation in JB6 mouse epidermal cells. Proc Natl Acad Sci U S A (1994);
18. Young M. R. *et al.* Transgenic mice demonstrate AP-1 (activator protein-1) transactivation is required for tumor promotion. Proc Natl Acad Sci U S A (1999);
19. Hsu T. C. *et al.* Transformation nonresponsive cells owe their resistance to lack of p65/nuclear factor-kappaB activation. Cancer Res (2001);
20. Hanahan D., Weinberg R. A. Hallmarks of cancer: the next generation. Cell (2011);
21. Halazonetis T. D. *et al.* An oncogene-induced DNA damage model for cancer development. Science (2008);
22. Negrini S. *et al.* Genomic instability--an evolving hallmark of cancer. Nat Rev Mol Cell Biol (2010);
23. Balkwill F., Mantovani A. Inflammation and cancer: back to Virchow? Lancet (2001);
24. Mantovani A. *et al.* Cancer-related inflammation. Nature (2008);
25. Zhang M. *et al.* A high M1/M2 ratio of tumor-associated macrophages is associated with extended survival in ovarian cancer patients. J Ovarian Res (2014);
26. Pantano F. *et al.* The role of macrophages polarization in predicting prognosis of radically resected gastric cancer patients. J Cell Mol Med (2013);
27. Ohri C. M. *et al.* Macrophages within NSCLC tumour islets are predominantly of a cytotoxic M1 phenotype associated with extended survival. Eur Respir J (2009);

28. Ma J. *et al.* The M1 form of tumor-associated macrophages in non-small cell lung cancer is positively associated with survival time. BMC Cancer (2010);
29. Bronkhorst I. H. *et al.* Detection of M2-macrophages in uveal melanoma and relation with survival. Invest Ophthalmol Vis Sci (2011);
30. Kurahara H. *et al.* Significance of M2-polarized tumor-associated macrophage in pancreatic cancer. J Surg Res (2011);
31. Garrido F. *et al.* The escape of cancer from T lymphocytes: immunoselection of MHC class I loss variants harboring structural-irreversible "hard" lesions. Cancer Immunol Immunother (2010);
32. Husain Z. *et al.* Tumor-derived lactate modifies antitumor immune response: effect on myeloid-derived suppressor cells and NK cells. J Immunol (2013);
33. Samani A. A. *et al.* The role of the IGF system in cancer growth and metastasis: overview and recent insights. Endocr Rev (2007);
34. Vivanco I., Sawyers C. L. The phosphatidylinositol 3-Kinase AKT pathway in human cancer. Nat Rev Cancer (2002);
35. Dick F. A., Rubin S. M. Molecular mechanisms underlying RB protein function. Nat Rev Mol Cell Biol (2013);
36. Debbas M., White E. Wild-type p53 mediates apoptosis by E1A, which is inhibited by E1B. Genes Dev (1993);
37. Serrano M. *et al.* Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. Cell (1997);
38. Hermeking H., Eick D. Mediation of c-Myc-induced apoptosis by p53. Science (1994);
39. Cross S. M. *et al.* A p53-dependent mouse spindle checkpoint. Science (1995);
40. Kastan M. B. *et al.* Participation of p53 protein in the cellular response to DNA damage. Cancer Res (1991);
41. Linke S. P. *et al.* A reversible, p53-dependent G0/G1 cell cycle arrest induced by ribonucleotide depletion in the absence of detectable DNA damage. Genes Dev (1996);
42. Di Leonardo A. *et al.* DNA damage triggers a prolonged p53-dependent G1 arrest and long-term induction of Cip1 in normal human fibroblasts. Genes Dev (1994);
43. Xie S. *et al.* Reactive oxygen species-induced phosphorylation of p53 on serine 20 is mediated in part by polo-like kinase-3. J Biol Chem (2001);
44. Hussain S. P., Harris C. C. p53 biological network: at the crossroads of the cellular-stress response pathway and molecular carcinogenesis. J Nippon Med Sch (2006);
45. Adams J. M., Cory S. The Bcl-2 apoptotic switch in cancer development and therapy. Oncogene (2007);
46. Westphal D. *et al.* Molecular biology of Bax and Bak activation and action. Biochim Biophys Acta (2011);
47. Phatak P., Burger A. M. Telomerase and its potential for therapeutic intervention. Br J Pharmacol (2007);
48. Weis S. M., Cheresh D. A. Tumor angiogenesis: molecular pathways and therapeutic targets. Nat Med (2011);
49. Shweiki D. *et al.* Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. Nature (1992);
50. Forsythe J. A. *et al.* Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. Mol Cell Biol (1996);
51. Mizukami Y. *et al.* Induction of interleukin-8 preserves the angiogenic response in HIF-1alpha-deficient colon cancer cells. Nat Med (2005);
52. Murdoch C. *et al.* The role of myeloid cells in the promotion of tumour angiogenesis. Nat. Rev. Cancer (2008);
53. Leek R. D. *et al.* Association of macrophage infiltration with angiogenesis and prognosis in invasive breast carcinoma. Cancer Res (1996);

54. Onita T. *et al.* Hypoxia-induced, perinecrotic expression of endothelial Per-ARNT-Sim domain protein-1/hypoxia-inducible factor-2alpha correlates with tumor progression, vascularization, and focal macrophage infiltration in bladder cancer. Clin Cancer Res (2002);
55. Takanami I. *et al.* Tumor-associated macrophage infiltration in pulmonary adenocarcinoma: association with angiogenesis and poor prognosis. Oncology (1999);
56. Kimura Y. N. *et al.* Inflammatory stimuli from macrophages and cancer cells synergistically promote tumor growth and angiogenesis. Cancer Sci (2007);
57. Chaffer C. L., Weinberg R. A. A perspective on cancer cell metastasis. Science (2011);
58. Wells A. *et al.* Epithelial and mesenchymal phenotypic switchings modulate cell motility in metastasis. Front Biosci (2011);
59. Umbas R. *et al.* Expression of the cellular adhesion molecule E-cadherin is reduced or absent in high-grade prostate cancer. Cancer Res (1992);
60. Mohri Y. Prognostic significance of E-cadherin expression in human colorectal cancer tissue. Surg Today (1997);
61. Dorudi S. *et al.* E-cadherin expression in colorectal cancer. An immunocytochemical and in situ hybridization study. Am J Pathol (1993);
62. Wenger R. H. *et al.* Integration of oxygen signaling at the consensus HRE. Sci STKE (2005);
63. Simon M. C., Keith B. The role of oxygen availability in embryonic development and stem cell function. Nat Rev Mol Cell Biol (2008);
64. Brown J. M., Wilson W. R. Exploiting tumour hypoxia in cancer treatment. Nat Rev Cancer (2004);
65. Hockel M. *et al.* Intratumoral pO2 predicts survival in advanced cancer of the uterine cervix. Radiother Oncol (1993);
66. Brizel D. M. *et al.* Tumor hypoxia adversely affects the prognosis of carcinoma of the head and neck. Int J Radiat Oncol Biol Phys (1997);
67. Nordsmark M. *et al.* Hypoxia in human soft tissue sarcomas: adverse impact on survival and no association with p53 mutations. Br J Cancer (2001);
68. Mayer A. *et al.* Endogenous hypoxia markers: case not proven! Adv Exp Med Biol (2008);
69. Carreau A. *et al.* Why is the partial oxygen pressure of human tissues a crucial parameter? Small molecules and hypoxia. J Cell Mol Med (2011);
70. Vordermark D. *et al.* Characterization of carbonic anhydrase IX (CA IX) as an endogenous marker of chronic hypoxia in live human tumor cells. Int J Radiat Oncol Biol Phys (2005);
71. Mekhail K. *et al.* HIF activation by pH-dependent nucleolar sequestration of VHL. Nat Cell Biol (2004);
72. Boado R. J., Pardridge W. M. Glucose deprivation and hypoxia increase the expression of the GLUT1 glucose transporter via a specific mRNA cis-acting regulatory element. J Neurochem (2002);
73. Evans S. M. *et al.* Detection of hypoxia in human squamous cell carcinoma by EF5 binding. Cancer Res (2000);
74. Varia M. A. *et al.* Pimonidazole: a novel hypoxia marker for complementary study of tumor hypoxia and cell proliferation in cervical carcinoma. Gynecol Oncol (1998);
75. Kaanders J. H. *et al.* Pimonidazole binding and tumor vascularity predict for treatment outcome in head and neck cancer. Cancer Res (2002);
76. Bristow R. G., Hill R. P. Hypoxia and metabolism. Hypoxia, DNA repair and genetic instability. Nat Rev Cancer (2008);
77. Wuest M., Wuest F. Positron emission tomography radiotracers for imaging hypoxia. J Labelled Comp Radiopharm (2013);

78. Price J. M. *et al.* Imaging hypoxia in tumours with advanced MRI. Q J Nucl Med Mol Imaging (2013);
79. Boron W. F., Boulpaep, E.L. Medical physiology, Saunders (2012);
80. Wenger R. H., Hoogewijs D. Regulated oxygen sensing by protein hydroxylation in renal erythropoietin-producing cells. Am J Physiol Renal Physiol (2010);
81. Kaelin W. G., Jr., Ratcliffe P. J. Oxygen sensing by metazoans: the central role of the HIF hydroxylase pathway. Mol Cell (2008);
82. Semenza G. L., Wang G. L. A nuclear factor induced by hypoxia via de novo protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation. Mol Cell Biol (1992);
83. Wiesener M. S. *et al.* Induction of endothelial PAS domain protein-1 by hypoxia: characterization and comparison with hypoxia-inducible factor-1alpha. Blood (1998);
84. Wiesener M. S. *et al.* Widespread hypoxia-inducible expression of HIF-2alpha in distinct cell populations of different organs. Faseb J (2003);
85. Tian H. *et al.* Endothelial PAS domain protein 1 (EPAS1), a transcription factor selectively expressed in endothelial cells. Genes Dev (1997);
86. Heikkila M. *et al.* Roles of the human hypoxia-inducible factor (HIF)-3alpha variants in the hypoxia response. Cell Mol Life Sci (2011);
87. Epstein A. C. *et al.* C. elegans EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. Cell (2001);
88. Ivan M. *et al.* HIFalpha targeted for VHL-mediated destruction by proline hydroxylation: implications for O2 sensing. Science (2001);
89. Masson N. *et al.* Independent function of two destruction domains in hypoxia-inducible factor-alpha chains activated by prolyl hydroxylation. Embo J (2001);
90. Chan D. A. *et al.* Role of prolyl hydroxylation in oncogenically stabilized hypoxia-inducible factor-1alpha. J Biol Chem (2002);
91. Kondo K. *et al.* Inhibition of HIF2alpha is sufficient to suppress pVHL-defective tumor growth. PLoS Biol (2003);
92. Takeda K. *et al.* Placental but not heart defects are associated with elevated hypoxia-inducible factor alpha levels in mice lacking prolyl hydroxylase domain protein 2. Mol Cell Biol (2006);
93. Takeda K. *et al.* Regulation of adult erythropoiesis by prolyl hydroxylase domain proteins. Blood (2008);
94. Koivunen P. *et al.* An endoplasmic reticulum transmembrane prolyl 4-hydroxylase is induced by hypoxia and acts on hypoxia-inducible factor alpha. J Biol Chem (2007);
95. Laitala A. *et al.* Transmembrane prolyl 4-hydroxylase is a fourth prolyl 4-hydroxylase regulating EPO production and erythropoiesis. Blood (2012);
96. Lando D. *et al.* Asparagine hydroxylation of the HIF transactivation domain a hypoxic switch. Science (2002);
97. Zhang N. *et al.* The asparaginyl hydroxylase factor inhibiting HIF-1alpha is an essential regulator of metabolism. Cell Metab (2010);
98. Kotch L. E. *et al.* Defective vascularization of HIF-1alpha-null embryos is not associated with VEGF deficiency but with mesenchymal cell death. Dev Biol (1999);
99. Kozak K. R. *et al.* ARNT-deficient mice and placental differentiation. Dev Biol (1997);
100. Maltepe E. *et al.* Abnormal angiogenesis and responses to glucose and oxygen deprivation in mice lacking the protein ARNT. Nature (1997);
101. Adelman D. M. *et al.* Multilineage embryonic hematopoiesis requires hypoxic ARNT activity. Genes Dev (1999);
102. Peng J. *et al.* The transcription factor EPAS-1/hypoxia-inducible factor 2alpha plays an important role in vascular remodeling. Proc Natl Acad Sci U S A (2000);
103. Compernolle V. *et al.* Loss of HIF-2alpha and inhibition of VEGF impair fetal lung maturation, whereas treatment with VEGF prevents fatal respiratory distress in premature mice. Nat Med (2002);

104. Tian H. *et al.* The hypoxia-responsive transcription factor EPAS1 is essential for catecholamine homeostasis and protection against heart failure during embryonic development. Genes Dev (1998);
105. Schodel J. *et al.* High-resolution genome-wide mapping of HIF-binding sites by ChIP-seq. Blood (2011);
106. Schofield C. J., Ratcliffe P. J. Oxygen sensing by HIF hydroxylases. Nat Rev Mol Cell Biol (2004);
107. Thomlinson R. H., Gray L. H. The histological structure of some human lung cancers and the possible implications for radiotherapy. Br J Cancer (1955);
108. Kallman R. F. The phenomenon of reoxygenation and its implications for fractionated radiotherapy. Radiology (1972);
109. Kim J. W., Dang C. V. Cancer's molecular sweet tooth and the Warburg effect. Cancer Res (2006);
110. Ebert B. L. *et al.* Hypoxia and mitochondrial inhibitors regulate expression of glucose transporter-1 via distinct Cis-acting sequences. J Biol Chem (1995);
111. Firth J. D. *et al.* Oxygen-regulated control elements in the phosphoglycerate kinase 1 and lactate dehydrogenase A genes: similarities with the erythropoietin 3' enhancer. Proc Natl Acad Sci U S A (1994);
112. Firth J. D. *et al.* Hypoxic regulation of lactate dehydrogenase A. Interaction between hypoxia-inducible factor 1 and cAMP response elements. J Biol Chem (1995);
113. Kim J. W. *et al.* HIF-1-mediated expression of pyruvate dehydrogenase kinase: a metabolic switch required for cellular adaptation to hypoxia. Cell Metab (2006);
114. Semenza G. L. *et al.* Hypoxia response elements in the aldolase A, enolase 1, and lactate dehydrogenase A gene promoters contain essential binding sites for hypoxia-inducible factor 1. J Biol Chem (1996);
115. Thamotharan S. *et al.* Hypoxic adaptation engages the CBP/CREST-induced coactivator complex of Creb-HIF-1alpha in transactivating murine neuroblastic glucose transporter. Am J Physiol Endocrinol Metab (2013);
116. Wykoff C. C. *et al.* Hypoxia-inducible expression of tumor-associated carbonic anhydrases. Cancer Res (2000);
117. Chiche J. *et al.* Hypoxia-inducible carbonic anhydrase IX and XII promote tumor cell growth by counteracting acidosis through the regulation of the intracellular pH. Cancer Res (2009);
118. Umbrello M. *et al.* The key role of nitric oxide in hypoxia: hypoxic vasodilation and energy supply-demand matching. Antioxid Redox Signal (2013);
119. Mukherjee D. Selective cyclooxygenase-2 (COX-2) inhibitors and potential risk of cardiovascular events. Biochem Pharmacol (2002);
120. Xue X., Shah Y. M. Hypoxia-inducible factor-2alpha is essential in activating the COX2/mPGES-1/PGE2 signaling axis in colon cancer. Carcinogenesis (2013);
121. Takeda N. *et al.* Endothelial PAS domain protein 1 gene promotes angiogenesis through the transactivation of both vascular endothelial growth factor and its receptor, Flt-1. Circ Res (2004);
122. Elvert G. *et al.* Cooperative interaction of hypoxia-inducible factor-2alpha (HIF-2alpha) and Ets-1 in the transcriptional activation of vascular endothelial growth factor receptor-2 (Flk-1). J Biol Chem (2003);
123. Simon M. P. *et al.* The angiopoietin-2 gene of endothelial cells is up-regulated in hypoxia by a HIF binding site located in its first intron and by the central factors GATA-2 and Ets-1. J Cell Physiol (2008);
124. Hu B., Cheng S. Y. Angiopoietin-2: development of inhibitors for cancer therapy. Curr Oncol Rep (2009);
125. Schito L. *et al.* Hypoxia-inducible factor 1-dependent expression of platelet-derived growth factor B promotes lymphatic metastasis of hypoxic breast cancer cells. Proc Natl Acad Sci U S A (2012);

126. Abramsson A. *et al.* Endothelial and nonendothelial sources of PDGF-B regulate pericyte recruitment and influence vascular pattern formation in tumors. J Clin Invest (2003);
127. Krishnamachary B. *et al.* Regulation of colon carcinoma cell invasion by hypoxia-inducible factor 1. Cancer Res (2003);
128. Yang S. *et al.* Hypoxia-inducible factor-2alpha is a catabolic regulator of osteoarthritic cartilage destruction. Nat Med (2010);
129. Itoh T. *et al.* Reduced angiogenesis and tumor progression in gelatinase A-deficient mice. Cancer Res (1998);
130. Bekes E. M. *et al.* Tumor-recruited neutrophils and neutrophil TIMP-free MMP-9 regulate coordinately the levels of tumor angiogenesis and efficiency of malignant cell intravasation. Am J Pathol (2011);
131. Rundhaug J. E. Matrix metalloproteinases and angiogenesis. J Cell Mol Med (2005);
132. Büchler P. *et al.* Transcriptional regulation of urokinase-type plasminogen activator receptor by hypoxia-inducible factor 1 is crucial for invasion of pancreatic and liver cancer. Neoplasia (2009);
133. Prager G. W. *et al.* Vascular endothelial growth factor receptor-2-induced initial endothelial cell migration depends on the presence of the urokinase receptor. Circ Res (2004);
134. Chin K. *et al.* Induction of vascular endothelial growth factor by nitric oxide in human glioblastoma and hepatocellular carcinoma cells. Oncogene (1997);
135. Ben-Av P. *et al.* Induction of vascular endothelial growth factor expression in synovial fibroblasts by prostaglandin E and interleukin-1: a potential mechanism for inflammatory angiogenesis. FEBS Lett (1995);
136. Ambs S. *et al.* p53 and vascular endothelial growth factor regulate tumor growth of NOS2-expressing human carcinoma cells. Nat Med (1998);
137. Dulak J. *et al.* Nitric oxide induces the synthesis of vascular endothelial growth factor by rat vascular smooth muscle cells. Arterioscler Thromb Vasc Biol (2000);
138. Kim K. S. *et al.* A novel role of hypoxia-inducible factor in cobalt chloride- and hypoxia-mediated expression of IL-8 chemokine in human endothelial cells. J Immunol (2006);
139. Li A. *et al.* IL-8 directly enhanced endothelial cell survival, proliferation, and matrix metalloproteinases production and regulated angiogenesis. J Immunol (2003);
140. Du R. *et al.* HIF1alpha induces the recruitment of bone marrow-derived vascular modulatory cells to regulate tumor angiogenesis and invasion. Cancer Cell (2008);
141. Feldser D. *et al.* Reciprocal positive regulation of hypoxia-inducible factor 1alpha and insulin-like growth factor 2. Cancer Res (1999);
142. Koshiji M. *et al.* HIF-1alpha induces cell cycle arrest by functionally counteracting Myc. Embo J (2004);
143. Gordan J. D. *et al.* HIF-2alpha promotes hypoxic cell proliferation by enhancing c-myc transcriptional activity. Cancer Cell (2007);
144. Bruick R. K. Expression of the gene encoding the proapoptotic Nip3 protein is induced by hypoxia. Proc Natl Acad Sci U S A (2000);
145. Guo K. *et al.* Hypoxia induces the expression of the pro-apoptotic gene BNIP3. Cell Death Differ (2001);
146. Greenhough A. *et al.* The COX-2/PGE2 pathway: key roles in the hallmarks of cancer and adaptation to the tumour microenvironment. Carcinogenesis (2009);
147. Erler J. T. *et al.* Lysyl oxidase is essential for hypoxia-induced metastasis. Nature (2006);
148. Schietke R. *et al.* The lysyl oxidases LOX and LOXL2 are necessary and sufficient to repress E-cadherin in hypoxia: insights into cellular transformation processes mediated by HIF-1. J Biol Chem (2010);
149. Evans A. J. *et al.* VHL promotes E2 box-dependent E-cadherin transcription by HIF-mediated regulation of SIP1 and snail. Mol Cell Biol (2007);

150. Buchler P. *et al.* Transcriptional regulation of urokinase-type plasminogen activator receptor by hypoxia-inducible factor 1 is crucial for invasion of pancreatic and liver cancer. Neoplasia (2009);
151. Masson V. *et al.* Contribution of host MMP-2 and MMP-9 to promote tumor vascularization and invasion of malignant keratinocytes. Faseb J (2005);
152. Phillips R. J. *et al.* Epidermal growth factor and hypoxia-induced expression of CXC chemokine receptor 4 on non-small cell lung cancer cells is regulated by the phosphatidylinositol 3-kinase/PTEN/AKT/mammalian target of rapamycin signaling pathway and activation of hypoxia inducible factor-1alpha. J Biol Chem (2005);
153. Ceradini D. J. *et al.* Progenitor cell trafficking is regulated by hypoxic gradients through HIF-1 induction of SDF-1. Nat Med (2004);
154. Wendel C. *et al.* CXCR4/CXCL12 participate in extravasation of metastasizing breast cancer cells within the liver in a rat model. PLoS One (2012);
155. Domanska U. M. *et al.* A review on CXCR4/CXCL12 axis in oncology: no place to hide. Eur J Cancer (2013);
156. Staller P. *et al.* Chemokine receptor CXCR4 downregulated by von Hippel-Lindau tumour suppressor pVHL. Nature (2003);
157. Koshiji M. *et al.* HIF-1alpha induces genetic instability by transcriptionally downregulating MutSalpha expression. Mol Cell (2005);
158. Lala P. K., Chakraborty C. Role of nitric oxide in carcinogenesis and tumour progression. Lancet Oncol (2001);
159. Bardos J. I., Ashcroft M. Hypoxia-inducible factor-1 and oncogenic signalling. Bioessays (2004);
160. Zhong H. *et al.* Overexpression of hypoxia-inducible factor 1alpha in common human cancers and their metastases. Cancer Res (1999);
161. Bertout J. A. *et al.* The impact of O2 availability on human cancer. Nat Rev Cancer (2008);
162. Baba Y. *et al.* HIF1A overexpression is associated with poor prognosis in a cohort of 731 colorectal cancers. Am. J. Pathol. (2010);
163. Gordan J. D. *et al.* HIF-alpha effects on c-Myc distinguish two subtypes of sporadic VHL-deficient clear cell renal carcinoma. Cancer Cell (2008);
164. Raval R. R. *et al.* Contrasting properties of hypoxia-inducible factor 1 (HIF-1) and HIF-2 in von Hippel-Lindau-associated renal cell carcinoma. Mol Cell Biol (2005);
165. Murphy K. M. *et al.* Janeway's Immunobiology, Taylor & Francis Ltd. (2008);
166. Gourko H., Williamson, D.I., Tauber, A.I. The Evolutionary Biology Papers of Elie Metchnikoff, Springer (2000);
167. Sakaguchi S. Naturally arising CD4+ regulatory t cells for immunologic self-tolerance and negative control of immune responses. Annu Rev Immunol (2004);
168. Sen R., Baltimore D. Multiple nuclear factors interact with the immunoglobulin enhancer sequences. Cell (1986);
169. Sen R., Baltimore D. Inducibility of kappa immunoglobulin enhancer-binding protein Nf-kappa B by a posttranslational mechanism. Cell (1986);
170. Perkins N. D., Gilmore T. D. Good cop, bad cop: the different faces of NF-kappaB. Cell Death Differ (2006);
171. Dajee M. *et al.* NF-kappaB blockade and oncogenic Ras trigger invasive human epidermal neoplasia. Nature (2003);
172. Hayden M. S., Ghosh S. NF-kappaB, the first quarter-century: remarkable progress and outstanding questions. Genes Dev (2012);
173. Oeckinghaus A., Ghosh S. The NF-kappaB family of transcription factors and its regulation. Cold Spring Harb Perspect Biol (2009);
174. Beg A. A. *et al.* Embryonic lethality and liver degeneration in mice lacking the RelA component of NF-kappa B. Nature (1995);

175. Sha W. C. *et al.* Targeted disruption of the p50 subunit of NF-kappa B leads to multifocal defects in immune responses. Cell (1995);
176. Kato T., Jr. *et al.* CK2 Is a C-Terminal IkappaB Kinase Responsible for NF-kappaB Activation during the UV Response. Mol Cell (2003);
177. Romieu-Mourez R. *et al.* Protein kinase CK2 promotes aberrant activation of nuclear factor-kappaB, transformed phenotype, and survival of breast cancer cells. Cancer Res (2002);
178. Rosetto M. *et al.* Signals from the IL-1 receptor homolog, Toll, can activate an immune response in a Drosophila hemocyte cell line. Biochem Biophys Res Commun (1995);
179. Medzhitov R. *et al.* A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. Nature (1997);
180. Kawai T., Akira S. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nat Immunol (2010);
181. Wu B. *et al.* Domain combination of the vertebrate-like TLR gene family: implications for their origin and evolution. J Genet
182. Gottipati S. *et al.* IRAK1: a critical signaling mediator of innate immunity. Cell Signal (2008);
183. Chang M. *et al.* Peli1 facilitates TRIF-dependent Toll-like receptor signaling and proinflammatory cytokine production. Nat Immunol (2009);
184. Cusson-Hermance N. *et al.* Rip1 mediates the Trif-dependent toll-like receptor 3- and 4-induced NF-{kappa}B activation but does not contribute to interferon regulatory factor 3 activation. J Biol Chem (2005);
185. Chen N. J. *et al.* Beyond tumor necrosis factor receptor: TRADD signaling in toll-like receptors. Proc Natl Acad Sci U S A (2008);
186. Jiang Z. *et al.* Toll-like receptor 3-mediated activation of NF-kappaB and IRF3 diverges at Toll-IL-1 receptor domain-containing adapter inducing IFN-beta. Proc Natl Acad Sci U S A (2004);
187. Kawai T., Akira S. TLR signaling. Semin Immunol (2007);
188. Fitzgerald K. A. *et al.* IKKepsilon and TBK1 are essential components of the IRF3 signaling pathway. Nat Immunol (2003);
189. Hemmi H. *et al.* The roles of two IkappaB kinase-related kinases in lipopolysaccharide and double stranded RNA signaling and viral infection. J Exp Med (2004);
190. Hacker H. *et al.* Specificity in Toll-like receptor signalling through distinct effector functions of TRAF3 and TRAF6. Nature (2006);
191. Lu Y. C. *et al.* LPS/TLR4 signal transduction pathway. Cytokine (2008);
192. Ye H. *et al.* Distinct molecular mechanism for initiating TRAF6 signalling. Nature (2002);
193. Deng L. *et al.* Activation of the IkappaB kinase complex by TRAF6 requires a dimeric ubiquitin-conjugating enzyme complex and a unique polyubiquitin chain. Cell (2000);
194. Kanayama A. *et al.* TAB2 and TAB3 activate the NF-kappaB pathway through binding to polyubiquitin chains. Mol Cell (2004);
195. Walsh M. C. *et al.* TRAF6 autoubiquitination-independent activation of the NFkappaB and MAPK pathways in response to IL-1 and RANKL. PLoS One (2008);
196. Conze D. B. *et al.* Lys63-linked polyubiquitination of IRAK-1 is required for interleukin-1 receptor- and toll-like receptor-mediated NF-kappaB activation. Mol Cell Biol (2008);
197. Xia Z. P. *et al.* Direct activation of protein kinases by unanchored polyubiquitin chains. Nature (2009);
198. Ni C. Y. *et al.* Cutting edge: K63-linked polyubiquitination of NEMO modulates TLR signaling and inflammation in vivo. J Immunol (2008);
199. Abbott D. W. *et al.* Coordinated regulation of Toll-like receptor and NOD2 signaling by K63-linked polyubiquitin chains. Mol Cell Biol (2007);
200. Tseng P. H. *et al.* Different modes of ubiquitination of the adaptor TRAF3 selectively activate the expression of type I interferons and proinflammatory cytokines. Nat Immunol (2010);

201. Harhaj E. W., Dixit V. M. Regulation of NF-kappaB by deubiquitinases. Immunol Rev (2012);
202. Pasparakis M. Role of NF-kappaB in epithelial biology. Immunol Rev (2012);
203. Colgan S. P., Taylor C. T. Hypoxia: an alarm signal during intestinal inflammation. Nat Rev Gastroenterol Hepatol (2010);
204. Bevins C. L., Salzman N. H. Paneth cells, antimicrobial peptides and maintenance of intestinal homeostasis. Nat Rev Microbiol (2011);
205. Nenci A. *et al.* Epithelial NEMO links innate immunity to chronic intestinal inflammation. Nature (2007);
206. Guma M. *et al.* Constitutive intestinal NF-kappaB does not trigger destructive inflammation unless accompanied by MAPK activation. J Exp Med (2011);
207. Vlantis K. *et al.* Constitutive IKK2 activation in intestinal epithelial cells induces intestinal tumors in mice. J Clin Invest (2011);
208. Eaden J. A. *et al.* The risk of colorectal cancer in ulcerative colitis: a meta-analysis. Gut (2001);
209. Canavan C. *et al.* Meta-analysis: colorectal and small bowel cancer risk in patients with Crohn's disease. Aliment Pharmacol Ther (2006);
210. Nikolaou K. *et al.* Inactivation of the deubiquitinase CYLD in hepatocytes causes apoptosis, inflammation, fibrosis, and cancer. Cancer Cell (2012);
211. Pflueger D. *et al.* Discovery of non-ETS gene fusions in human prostate cancer using next-generation RNA sequencing. Genome Res (2011);
212. Borrello M. G. *et al.* Induction of a proinflammatory program in normal human thyrocytes by the RET/PTC1 oncogene. Proc Natl Acad Sci U S A (2005);
213. Sparmann A., Bar-Sagi D. Ras-induced interleukin-8 expression plays a critical role in tumor growth and angiogenesis. Cancer Cell (2004);
214. Puvvada S. D. *et al.* NF-κB and Bcl-3 activation are prognostic in metastatic colorectal cancer. Oncology (2010);
215. Weichert W. *et al.* High expression of RelA/p65 is associated with activation of nuclear factor-kappaB-dependent signaling in pancreatic cancer and marks a patient population with poor prognosis. Br J Cancer (2007);
216. Zhang Y. *et al.* Association of nuclear factor kappaB expression with a poor outcome in nasopharyngeal carcinoma. Med Oncol (2010);
217. Guo R. X. *et al.* Increased staining for phosphorylated AKT and nuclear factor-kappaB p65 and their relationship with prognosis in epithelial ovarian cancer. Pathol Int (2008);
218. Izzo J. G. *et al.* Association of activated transcription factor nuclear factor kappab with chemoradiation resistance and poor outcome in esophageal carcinoma. J Clin Oncol (2006);
219. Jin X. *et al.* Potential biomarkers involving IKK/RelA signal in early stage non-small cell lung cancer. Cancer Sci (2008);
220. Li J. *et al.* Association of constitutive nuclear factor-kappaB activation with aggressive aspects and poor prognosis in cervical cancer. Int J Gynecol Cancer (2009);
221. Greten F. R. *et al.* IKKbeta links inflammation and tumorigenesis in a mouse model of colitis-associated cancer. Cell (2004);
222. Grivennikov S. I., Karin M. Dangerous liaisons: STAT3 and NF-kappaB collaboration and crosstalk in cancer. Cytokine Growth Factor Rev (2010);
223. Lu H. *et al.* Inflammation, a key event in cancer development. Mol Cancer Res (2006);
224. Grivennikov S. I. *et al.* Immunity, inflammation, and cancer. Cell (2010);
225. Yang J. *et al.* Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis. Cell (2004);
226. Li C. W. *et al.* Epithelial-mesenchymal transition induced by TNF-alpha requires NF-kappaB-mediated transcriptional upregulation of Twist1. Cancer Res (2012);

227. Chua H. L. *et al.* NF-kappaB represses E-cadherin expression and enhances epithelial to mesenchymal transition of mammary epithelial cells: potential involvement of ZEB-1 and ZEB-2. Oncogene (2007);
228. Morris K. R. *et al.* Role of the NF-kappaB signaling pathway and kappaB cis-regulatory elements on the IRF-1 and iNOS promoter regions in mycobacterial lipoarabinomannan induction of nitric oxide. Infect Immun (2003);
229. Bond M. *et al.* Synergistic upregulation of metalloproteinase-9 by growth factors and inflammatory cytokines: an absolute requirement for transcription factor NF-kappa B. FEBS Lett (1998);
230. Yamamoto K. *et al.* Transcriptional roles of nuclear factor kappa B and nuclear factor-interleukin-6 in the tumor necrosis factor alpha-dependent induction of cyclooxygenase-2 in MC3T3-E1 cells. J Biol Chem (1995);
231. Lee S. J. *et al.* High glucose enhances MMP-2 production in adventitial fibroblasts via Akt1-dependent NF-kappaB pathway. FEBS Lett (2007);
232. Galdiero M. R. *et al.* Tumor associated macrophages and neutrophils in tumor progression. J Cell Physiol (2013);
233. Lamagna C. *et al.* Dual role of macrophages in tumor growth and angiogenesis. J Leukoc Biol (2006);
234. Martinez F. O. *et al.* Macrophage activation and polarization. Front Biosci (2008);
235. Langowski J. L. *et al.* Swords into plowshares: IL-23 repurposes tumor immune surveillance. Trends Immunol (2007);
236. Liu C. *et al.* Act1, a U-box E3 ubiquitin ligase for IL-17 signaling. Sci Signal (2009);
237. Sica A. *et al.* Autocrine production of IL-10 mediates defective IL-12 production and NF-kappa B activation in tumor-associated macrophages. J Immunol (2000);
238. Fiorentino D. F. *et al.* Two types of mouse T helper cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. J Exp Med (1989);
239. Solinas G. *et al.* Tumor-associated macrophages (TAM) as major players of the cancer-related inflammation. J Leukoc Biol (2009);
240. Zamarron B. F., Chen W. Dual roles of immune cells and their factors in cancer development and progression. Int J Biol Sci (2011);
241. Galon J. *et al.* Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. Science (2006);
242. Tosolini M. *et al.* Clinical impact of different classes of infiltrating T cytotoxic and helper cells (Th1, th2, treg, th17) in patients with colorectal cancer. Cancer Res (2011);
243. Oleinika K. *et al.* Suppression, subversion and escape: the role of regulatory T cells in cancer progression. Clin Exp Immunol (2013);
244. Wang J. *et al.* The clinical significance of tumor-infiltrating neutrophils and neutrophil-to-CD8+ lymphocyte ratio in patients with resectable esophageal squamous cell carcinoma. J Transl Med (2014);
245. Rao H. L. *et al.* Increased intratumoral neutrophil in colorectal carcinomas correlates closely with malignant phenotype and predicts patients' adverse prognosis. PLoS One (2012);
246. Humphries A., Wright N. A. Colonic crypt organization and tumorigenesis. Nat Rev Cancer (2008);
247. Vaiopoulos A. G. *et al.* Colorectal cancer stem cells. Stem Cells (2012);
248. Barker N. *et al.* Crypt stem cells as the cells-of-origin of intestinal cancer. Nature (2009);
249. Burt R. Inheritance of Colorectal Cancer. Drug Discov Today Dis Mech (2007);
250. Mattar M. C. *et al.* Current management of inflammatory bowel disease and colorectal cancer. Gastrointest Cancer Res (2011);
251. Terzic J. *et al.* Inflammation and colon cancer. Gastroenterology (2010);
252. Compare D., Nardone G. The bacteria-hypothesis of colorectal cancer: pathogenetic and therapeutic implications. Translational Gastrointestinal Cancer (2014);

253. Vannucci L. *et al.* Colorectal carcinogenesis in germ-free and conventionally reared rats: different intestinal environments affect the systemic immunity. Int J Oncol (2008);
254. Santaolalla R. *et al.* TLR signaling: a link between gut microflora, colorectal inflammation and tumorigenesis. Drug Discovery Today: Disease Mechanisms (2011);
255. Fukata M. *et al.* Toll-like receptor-4 promotes the development of colitis-associated colorectal tumors. Gastroenterology (2007);
256. Wang E. L. *et al.* High expression of Toll-like receptor 4/myeloid differentiation factor 88 signals correlates with poor prognosis in colorectal cancer. Br. J. Cancer (2010);
257. Fukata M. *et al.* Constitutive activation of epithelial TLR4 augments inflammatory responses to mucosal injury and drives colitis-associated tumorigenesis. Inflamm Bowel Dis (2011);
258. Goethals L. *et al.* Hypoxia in human colorectal adenocarcinoma: comparison between extrinsic and potential intrinsic hypoxia markers. Int J Radiat Oncol Biol Phys (2006);
259. Lin G. *et al.* KRAS mutation and NF-kappaB activation indicates tolerance of chemotherapy and poor prognosis in colorectal cancer. Dig Dis Sci (2012);
260. Karhausen J. *et al.* Epithelial hypoxia-inducible factor-1 is protective in murine experimental colitis. J Clin Invest (2004);
261. Cramer T. *et al.* HIF-1alpha is essential for myeloid cell-mediated inflammation. Cell (2003);
262. Walmsley S. R. *et al.* Hypoxia-induced neutrophil survival is mediated by HIF-1alpha-dependent NF-kappaB activity. J Exp Med (2005);
263. Rius J. *et al.* NF-kappaB links innate immunity to the hypoxic response through transcriptional regulation of HIF-1alpha. Nature (2008);
264. van Uden P. *et al.* Regulation of hypoxia-inducible factor-1alpha by NF-kappaB. Biochem J (2008);
265. Bonello S. *et al.* Reactive oxygen species activate the HIF-1alpha promoter via a functional NFkappaB site. Arterioscler Thromb Vasc Biol (2007);
266. Blouin C. C. *et al.* Hypoxic gene activation by lipopolysaccharide in macrophages: implication of hypoxia-inducible factor 1alpha. Blood (2004);
267. Frede S. *et al.* Bacterial lipopolysaccharide induces HIF-1 activation in human monocytes via p44/42 MAPK and NF-kappaB. Biochem J (2006);
268. Belaiba R. S. *et al.* Hypoxia up-regulates hypoxia-inducible factor-1alpha transcription by involving phosphatidylinositol 3-kinase and nuclear factor kappaB in pulmonary artery smooth muscle cells. Mol Biol Cell (2007);
269. Zhou J. *et al.* Tumor necrosis factor-alpha causes accumulation of a ubiquitinated form of hypoxia inducible factor-1alpha through a nuclear factor-kappaB-dependent pathway. Mol Biol Cell (2003);
270. Jung Y. J. *et al.* IL-1beta-mediated up-regulation of HIF-1alpha via an NFkappaB/COX-2 pathway identifies HIF-1 as a critical link between inflammation and oncogenesis. FASEB J (2003);
271. Jung Y. *et al.* Hypoxia-inducible factor induction by tumour necrosis factor in normoxic cells requires receptor-interacting protein-dependent nuclear factor kappa B activation. Biochem J (2003);
272. van Uden P. *et al.* Evolutionary conserved regulation of HIF-1beta by NF-kappaB. PLoS Genet (2011);
273. Bracken C. P. *et al.* Activity of hypoxia-inducible factor 2alpha is regulated by association with the NF-kappaB essential modulator. J Biol Chem (2005);
274. Cummins E. P. *et al.* Prolyl hydroxylase-1 negatively regulates IkappaB kinase-beta, giving insight into hypoxia-induced NFkappaB activity. Proc Natl Acad Sci U S A (2006);
275. Culver C. *et al.* Mechanism of hypoxia-induced NF-kappaB. Mol Cell Biol (2010);

276. An J. *et al.* Inactivation of the CYLD deubiquitinase by HPV E6 mediates hypoxia-induced NF-kappaB activation. Cancer Cell (2008);
277. Scholz C. C. *et al.* Regulation of IL-1beta-induced NF-kappaB by hydroxylases links key hypoxic and inflammatory signaling pathways. Proc Natl Acad Sci U S A (2013);
278. Takeda K. *et al.* Inhibition of prolyl hydroxylase domain-containing protein suppressed lipopolysaccharide-induced TNF-alpha expression. Arterioscler Thromb Vasc Biol (2009);
279. Hu F. *et al.* Hypoxia and hypoxia-inducible factor-1alpha provoke toll-like receptor signalling-induced inflammation in rheumatoid arthritis. Ann Rheum Dis (2013);
280. Cummins E. P. *et al.* The hydroxylase inhibitor dimethyloxalylglycine is protective in a murine model of colitis. Gastroenterology (2008);
281. Tambuwala M. M. *et al.* Loss of prolyl hydroxylase-1 protects against colitis through reduced epithelial cell apoptosis and increased barrier function. Gastroenterology (2010);
282. Xue X. *et al.* Endothelial PAS Domain Protein 1 Activates the Inflammatory Response in the Intestinal Epithelium to Promote Colitis in Mice. Gastroenterology (2013);
283. Vavricka S. R. *et al.* High altitude journeys and flights are associated with an increased risk of flares in inflammatory bowel disease patients. J Crohns Colitis (2013);
284. Eltzschig H. K., Carmeliet P. Hypoxia and inflammation. N. Engl. J. Med. (2011);
285. Wenger R. H. *et al.* Integration of oxygen signaling at the consensus HRE. Sci. STKE (2005);
286. Pouysségur J. *et al.* Hypoxia signalling in cancer and approaches to enforce tumour regression. Nature (2006);
287. Bernstein C. N. *et al.* Cancer risk in patients with inflammatory bowel disease: a population-based study. Cancer (2001);
288. Mercurio F. *et al.* IKK-1 and IKK-2: cytokine-activated IκB kinases essential for NF-κB activation. Science (1997);
289. Dolcet X. *et al.* NF-κB in development and progression of human cancer. Virchows Arch. (2005);
290. Hirani N. *et al.* The regulation of interleukin-8 by hypoxia in human macrophages - a potential role in the pathogenesis of the acute respiratory distress syndrome (ARDS). Mol. Med. (2001);
291. Cummins E. P. *et al.* Prolyl hydroxylase-1 negatively regulates IκB kinase-β, giving insight into hypoxia-induced NFκB activity. Proc. Natl. Acad. Sci. U.S.A. (2006);
292. An J. *et al.* Inactivation of the CYLD deubiquitinase by HPV E6 mediates hypoxia-induced NF-κB activation. Cancer Cell (2008);
293. Culver C. *et al.* Mechanism of hypoxia-induced NF-κB. Mol. Cell. Biol. (2010);
294. Fitzpatrick S. F. *et al.* An intact canonical NF-κB pathway is required for inflammatory gene expression in response to hypoxia. J. Immunol. (2011);
295. Cockman M. E. *et al.* Posttranslational hydroxylation of ankyrin repeats in IκB proteins by the hypoxia-inducible factor (HIF) asparaginyl hydroxylase, factor inhibiting HIF (FIH). Proc. Natl. Acad. Sci. U.S.A. (2006);
296. Belaiba R. S. *et al.* Hypoxia up-regulates hypoxia-inducible factor-1α transcription by involving phosphatidylinositol 3-kinase and nuclear factor κB in pulmonary artery smooth muscle cells. Mol. Biol. Cell (2007);
297. Bonello S. *et al.* Reactive oxygen species activate the HIF-1α promoter via a functional NFκB site. Arterioscler. Thromb. Vasc. Biol. (2007);
298. Rius J. *et al.* NF-κB links innate immunity to the hypoxic response through transcriptional regulation of HIF-1α. Nature (2008);
299. van Uden P. *et al.* Regulation of hypoxia-inducible factor-1α by NF-κB. Biochem. J. (2008);

300. Yoshida T. *et al.* Transcriptional upregulation of HIF-1α by NF-κB/p65 and its associations with β-catenin/p300 complexes in endometrial carcinoma cells. Lab. Invest. (2013);
301. Gassmann M. *et al.* Oxygen supply and oxygen-dependent gene expression in differentiating embryonic stem cells. Proc. Natl. Acad. Sci. U.S.A. (1996);
302. Novell A. *et al.* Prognostic value of c-FLIPL/s, HIF-1α, and NF-κB in stage II and III rectal cancer. Virchows Arch. (2014);
303. Rawluszko-Wieczorek A. A. *et al.* Prognostic potential of DNA methylation and transcript levels of HIF1A and EPAS1 in colorectal cancer. Mol. Cancer Res. (2014);
304. Fu J., Taubman M. B. Prolyl hydroxylase EGLN3 regulates skeletal myoblast differentiation through an NF-κB-dependent pathway. J. Biol. Chem. (2010);
305. Takeda K. *et al.* Inhibition of prolyl hydroxylase domain-containing protein suppressed lipopolysaccharide-induced TNF-α expression. Arterioscler. Thromb. Vasc. Biol. (2009);
306. Schödel J. *et al.* High-resolution genome-wide mapping of HIF-binding sites by ChIP-seq. Blood (2011);
307. Scholz C. C. *et al.* Regulation of IL-1β-induced NF-κB by hydroxylases links key hypoxic and inflammatory signaling pathways. Proc. Natl. Acad. Sci. U.S.A. (2013);
308. Borsig L. *et al.* Synergistic effects of L- and P-selectin in facilitating tumor metastasis can involve non-mucin ligands and implicate leukocytes as enhancers of metastasis. Proc. Natl. Acad. Sci. U.S.A. (2002);
309. Chomczynski P., Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. (1987);
310. Mirsaidi A. *et al.* Preparation and osteogenic differentiation of scaffold-free mouse adipose-derived stromal cell microtissue spheroids (ASC-MT). Current protocols in stem cell biology (2013);
311. de Andres M. C. *et al.* Loss of methylation in CpG sites in the NF-kappaB enhancer elements of inducible nitric oxide synthase is responsible for gene induction in human articular chondrocytes. Arthritis Rheum (2013);
312. Bednarik D. P. *et al.* DNA CpG methylation inhibits binding of NF-kappa B proteins to the HIV-1 long terminal repeat cognate DNA motifs. New Biol (1991);
313. Koslowski M. *et al.* Tumor-associated CpG demethylation augments hypoxia-induced effects by positive autoregulation of HIF-1alpha. Oncogene (2011);
314. Yamashita S. *et al.* Chemical genomic screening for methylation-silenced genes in gastric cancer cell lines using 5-aza-2'-deoxycytidine treatment and oligonucleotide microarray. Cancer Sci (2006);
315. Juttermann R. *et al.* Toxicity of 5-aza-2'-deoxycytidine to mammalian cells is mediated primarily by covalent trapping of DNA methyltransferase rather than DNA demethylation. Proc Natl Acad Sci U S A (1994);
316. Santi D. V. *et al.* Covalent bond formation between a DNA-cytosine methyltransferase and DNA containing 5-azacytosine. Proc Natl Acad Sci U S A (1984);
317. Owen H. R. *et al.* Identification of novel and cell type enriched cofactors of the transcription activation domain of RelA (p65 NF-kappaB). J Proteome Res (2005);
318. Bosetti C. *et al.* Aspirin and cancer risk: a summary review to 2007. Recent Results Cancer Res (2009);
319. Garcia-Rodriguez L. A., Huerta-Alvarez C. Reduced risk of colorectal cancer among long-term users of aspirin and nonaspirin nonsteroidal antiinflammatory drugs. Epidemiology (2001);
320. Flossmann E., Rothwell P. M. Effect of aspirin on long-term risk of colorectal cancer: consistent evidence from randomised and observational studies. Lancet (2007);
321. Jaakkola P. *et al.* Targeting of HIF-α to the von Hippel-Lindau ubiquitylation complex by O_2-regulated prolyl hydroxylation. Science (2001);

9. Acknowledgements

I would like to thank Prof. Roland H. Wenger who gave me the opportunity to perform my PhD thesis in his lab and took care of my scientific development during that time. I have received also a lot of support and attention from my other PhD thesis committee members Prof. Michel O. Hottiger and PD Dr. Lubor Borsig, and hereby I thank them for their positive influence on the quality of my work. I would also like to thank my supervisor Dr. David Hoogewijs, especially for his kind way of expressing opinion on my performance.

Karolin Léger, Carole Oertli and Jesus Glaus, who participated in the ProDoc project, I would like to thank for support, friendliness and hard work during our joint research years.

Many thanks go to Patrick Spielmann, for working, crying, hoping and laughing together.

A great influence on the everyday lab atmosphere had Irene A. Rodríguez, Margot Crucet, Alexandra Schörg, Federica Storti and Sascha Egger, all of whom I would like to kindly thank for their open, straightforward and helpful attitude.

Furthermore, I would like to thank all other current and former members of our lab for the time spent together.

Special thanks go to Björn for his constant strong support over the past few years.